



## **Seroepidemiology of *Coxiella burnetii* in Danish Cattle**

Paul, Suman

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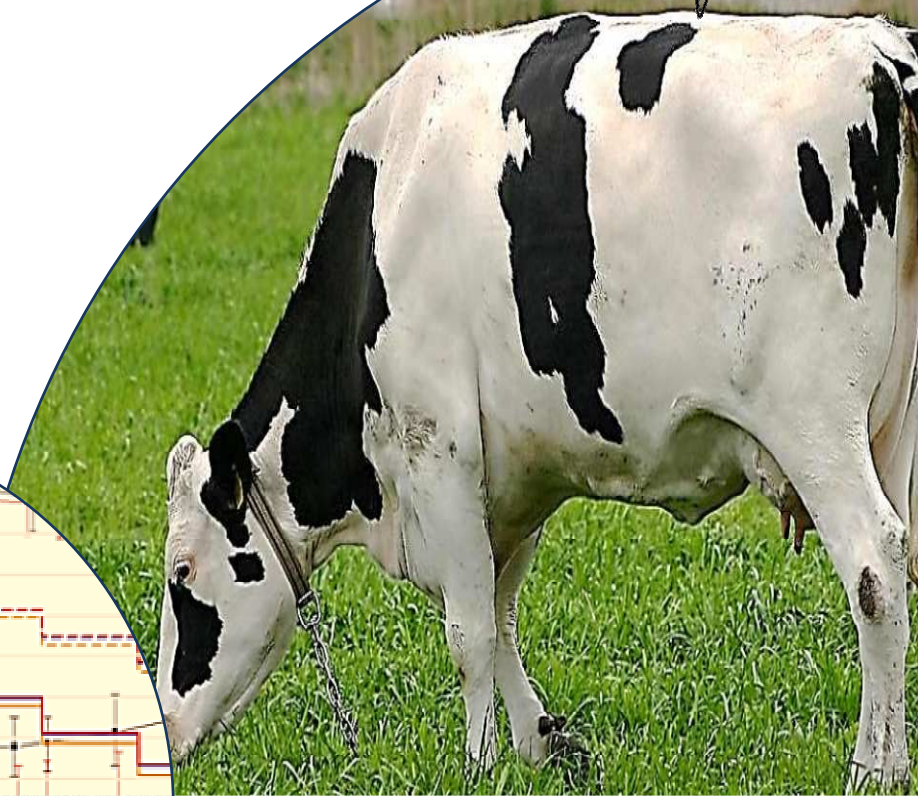
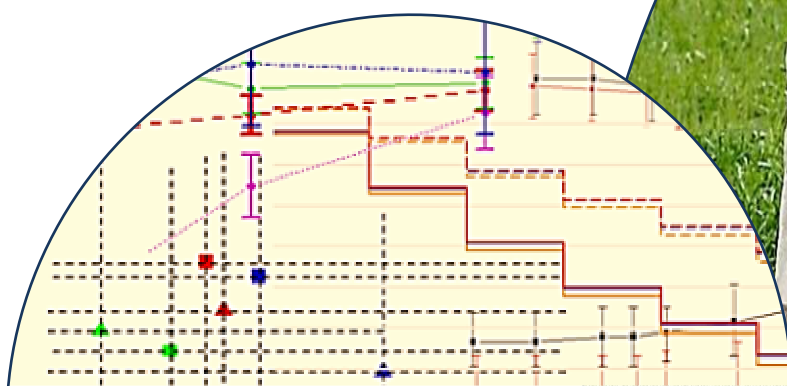
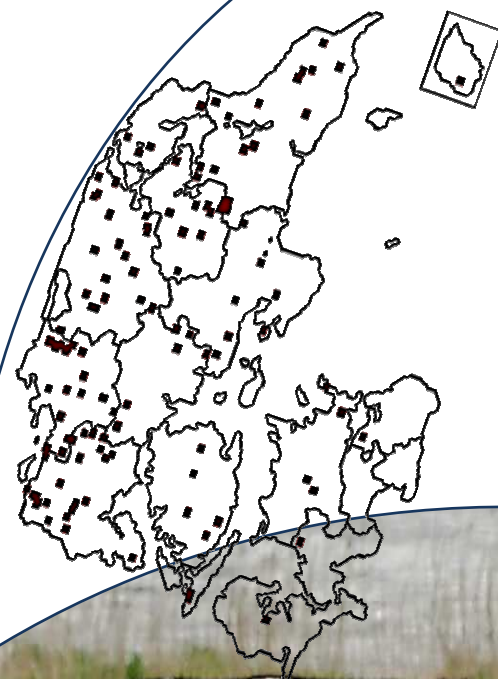
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SUMAN PAUL | PHD THESIS 2013

# Seroepidemiology of *Coxiella burnetii* in Danish Cattle





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This thesis has been submitted to the Graduate School of The Faculty of Health and Medical Sciences, University of Copenhagen.

**SUMAN PAUL  
PhD Thesis 2013**

**Faculty of Health and Medical Sciences  
Department of Large Animal Sciences  
University of Copenhagen  
Denmark**

## **SUPERVISORS**

### **Principal supervisor**

**Associate Professor Jens Frederik Gramstrup Agger**, Section of Population Biology, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

### **Co-supervisors**

**Professor Jørgen Steen Agerholm**, Section of Veterinary Reproduction and Obstetrics, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

**Associate Professor Bo Markussen**, Laboratory of Applied Statistics, Department of Mathematical Sciences, Faculty of Science, University of Copenhagen, Denmark.

## **ASSESSMENT COMMITTEE**

**Professor Liza Rosenbaum Nielsen**, Section of Population Biology, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

**Professor Francois Beaudeau**, Quantitative Veterinary Epidemiology, ONIRIS (Nantes Atlantic National college of Veterinary Medicine, Food Science and Engineering), Site de la Chantrerie, CS 40706-44307 Nantes cedex 03, France.

**Associate Professor Håkan Vigre**, Technical University of Denmark, National Food Institute, Mørkhøj Bygade 19, Building H, 2860 Søborg, Denmark.

## **Seroepidemiology of *Coxiella burnetii* in Danish Cattle**

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## **ISBN**

## **Dedication**

**For my parents, brother, wife and lovely kids**



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## **PREFACE**

This PhD project has been a deep, intensive and challenging learning process for me; both from a scientific and human point of view. During the last three years, I had great scientific and human support from my supervisors, colleagues, friends and family.

This thesis is the result of my three year PhD education at the Faculty of Health and Medical Sciences (formerly, Faculty of Life Sciences) of the University of Copenhagen. I acknowledge the University of Copenhagen for financing this project.

During the PhD process, I had the opportunity to work with and learn from excellent scientists and human beings. The whole PhD process was intensively supervised by Associate Professor Jens Frederik Agger. He has been an excellent supervisor for me and one of the most wonderful human beings I have ever met. I would like to express my sincere gratitude to him for his enormous generosity, commitment and inspiration. I was also honoured to have support from Professor Jørgen Steen Agerholm and Associate Professor Bo Markussen in developing my PhD plan and in different manuscripts. I would like to thank both of them for their contributions and for always being helpful.

I acknowledge the contributions of the Danish Knowledge Centre for Agriculture, especially Niels Ole Nielsen, Jørgen Nielsen and Erik Rattenborg for providing supplementary data for the studies. I also acknowledge Lisa Mejlvang Lindgaard and Dorte Thanning Lavritsen, Eurofins Steins, Holsterbro, Denmark for milk sample collection and good laboratory work.

I would also like to thank all of my colleagues in the group of Population Biology, Department of Large Animal Sciences, with special thanks to Jeanne Talchow Oakman for providing tremendous instrumental support that always came with a smile. Thanks to all of my fellow PhD colleagues for sharing their frustrations and joys. Thanks Marshal for helping in producing graphs in R and sharing scientific views at different occasions. Professor Nils Toft is thanked for helping me in two manuscripts and providing scientific advice when needed. Thanks to Anna-Bodil Christoffersen for her contributions in different manuscripts.

I must acknowledge the contributions of Syed Sayeem Uddin Ahmed, my friend and colleague, for his tremendous mental support during my entire PhD tenure. A special thanks to Himel Barua for the proofreading of this thesis. I gladly remember the help of Sharmin Chowdhury during my stay in Copenhagen. Thanks to all my fellow Bangladeshi PhDs at the Faculty of Health and Medical Sciences for making my life easy and enjoyable in Copenhagen.

Last but not least, I would like to thank my wife and my two lovely kids for your endless support in finishing my PhD project. I apologise for staying away from you for the last four months or so, and I promise not to let it happen again!

April 15, 2013

Suman Paul



## SUMMARY

*Coxiella burnetii*, the causative agent of Q fever in humans and animals, is an obligate intracellular bacterium with zoonotic potential. This bacterium has a worldwide distribution and can infect a wide range of hosts, including domestic ruminants. Like many other European countries, the presence of *C. burnetii* has been reported for several years in Denmark, and detection of the agent in animals in Denmark was made notifiable from December 2004. Since the recent *C. burnetii* epidemic in goats, sheep and man during 2007-2010 in the Netherlands, epidemiological research on Q fever in both animals and humans has gained more attention from researchers in many European countries, including Denmark. At that time, there was only limited information about its prevalence, the risk factors for *C. burnetii* infection, the dynamics of the infection and the validity of the tests used for the diagnosis of the infection or its seropositivity in cattle. Thus, the overall objectives of this thesis were to investigate the epidemiology of the *C. burnetii* antibody positivity in the Danish cattle population related to its frequency and risk factors, and also to evaluate the performance of the diagnostic tests used in identifying *C. burnetii* antibody positivity in animals or herds.

To reach these objectives, the thesis is based on a series of epidemiological studies using Danish data collected during 2008-2009 from 100 randomly selected dairy herds, during 2012-2013 from 120 randomly selected dairy herds, and in 2012 from 800 slaughtered cattle. The data comprised laboratory testing for the presence of *C. burnetii* antibodies in milk and blood samples from individual cattle, and in samples from bulk tank milk (BTM) of dairy herds. This data was supplemented with herd level data from questionnaire interviews with farmers and with relevant secondary animal and herd level data from the Danish Cattle Database.

A cross-sectional study including 120 randomly selected Danish dairy herds in July 2012, showed that BTM samples of 79% of herds were *C. burnetii* antibody positive, and this prevalence was significantly higher than that estimated in the study in 2008 (Chapter 3). An adverse effect of increasing herd size and average cattle density on the risk of seropositivity was identified in the same study.

To identify the risk factors for *C. burnetii* antibody positivity in Danish dairy cattle herds, BTM samples from 100 randomly selected herds were analysed in a cross-sectional study (Chapter 4a). To obtain the management and herd biosecurity information, farm managers were telephone interviewed about labour, biosecurity, housing and herd health during the 12 months prior to the study. Results showed that sharing of machines between farms (Yes vs. No, odds ratio (OR) =3.6), human contact (Yes vs. No, OR=4.2), insemination by people other than artificial insemination technicians (Yes vs. No, OR=7.7), a herd health contract with a veterinarian (Yes vs. No, OR=4.3) and improved hygiene precautions taken by veterinarians (No vs. Yes, OR=5) were significantly associated with antibody positivity. This study confirms that strict biosecurity procedures are important for the prevention of *C. burnetii* infection in dairy herds.

The study population of 24 stratified randomly selected dairy herds from 2008; along with cow level and herd level management information obtained from the Danish

Cattle Database and from a questionnaire survey was used to identify factors associated with *C. burnetii* antibody positivity in Danish dairy cows (Chapter 4b). Results of multivariable logistic regression analysis, accounting for hierarchical structures (cow and herd), showed that the risk of being seropositive was higher in the Danish Holstein breed than in other dairy breeds and during summer compared to other seasons. The risk increased with the increasing number of parity and high milk protein contents; but decreased with increasing milk yield and a high milk fat content. Herd size, tie stall housing systems, quarantine of newly purchased animals and good hygienic precautions taken by the veterinarian before entering the stable, were also significantly associated with reduced odds of *C. burnetii* antibody positivity.

Within herd prevalence of antibody positivity was also estimated in 24 selected dairy herds used in Chapter 4b. These herds comprised ten randomly sampled herds out of herds that tested positive on the BTM at the start of the study, ten randomly sampled herds among herds that tested negative on the BTM and four randomly sampled herds among herds that tested intermediate on the BTM (Chapter 4b). Results showed that the prevalence in initially BTM positive, intermediate and negative herds was 27%, 23% and 0.9%, respectively. Within herd prevalence, in initially BTM positive and BTM intermediate herds, remained stable at three sampling time points during an 11-month follow-up period. This indicated a long lasting stability of within herd seroprevalence. The prevalence increased significantly in initially BTM negative herds during the same study period. These changes in the within herd prevalence (Chapter 4b) indicated that the antibody status of some cows shifted from positive to negative, or vice versa during the study period.

Blood and milk samples from 568 lactating cows from 17 Danish dairy cattle herds collected in 2008 were used to determine the Se and Sp of an ELISA for detection of *C. burnetii* antibodies in milk and blood samples, using latent class models in a Bayesian analysis (Chapter 5). Based on the estimates of a differential positive rate (DPR), the best combination of the Se and Sp estimates was revealed at a sample to positive (S/P) cut-off of 40 for both blood and milk ELISAs. At this cut-off, the Se and Sp of milk ELISA were 0.86 and 0.99, respectively; whereas the Se and Sp of blood ELISA were 0.84 and 0.99, respectively. The difference between the Se estimates of these two tests was not statistically significant, indicating an equal diagnostic capability of these two test methods. Therefore, the choice of test can be based on the context of the study. For serological surveillance of lactating dairy cows, the milk ELISA test could be the preferred method, and the blood ELISA test can be useful for serological studies in non-lactating cattle. No conditional dependence was observed between the Sp estimates of the two test methods, and the Se estimates of both tests were significantly reduced only when a conditional covariance  $\geq 40$  was used. This implies that there is no absolute dependence between these two test methods, and hence, neither of them affects the Se and Sp estimates when comparisons are made between them. The Se and Sp of blood ELISA estimated in this study were used to calculate the true prevalence of *C. burnetii* seropositivity in Danish slaughter cattle.

Another cross-sectional serosurvey in slaughtered cattle was conducted to estimate the seroprevalence of *C. burnetii* in cattle raised for meat production (Chapter 6). The level of antibodies was analysed in blood samples from 800 randomly selected slaughter cattle from six major Danish slaughter houses between August and October 2012. Bayesian

models, allowing an adjustment for the test sensitivity (Se) and specificity (Sp), were used to estimate apparent prevalence (AP), true prevalence and true prevalence using a random effect model for different breeds (Chapter 6). The overall AP and TP in Danish slaughter cattle were 5.6%. Significant differences in the prevalence were observed between the slaughtered cattle of dairy (AP=9.1%; TP=9.5%) and beef breeds (AP=4.3%; TP=3.5%); between male (AP=3.6%; TP=2.6%) and female (AP=9.1%; TP=9.4%); and between dairy (AP=15.1%; TP=16.7%) and beef (AP=4.5%; TP=3.6%) herds that the cattle delivered from to slaughter. The results indicated that cattle sent to slaughter have a lower seroprevalence than lactating dairy cows; and among the slaughtered cattle, the majority of seropositives originated from dairy breeds. A Bayesian logistic regression model identified positive associations between *C. burnetii* seropositivity and age, breed type and number of movements between herds.

In conclusion, the results from this PhD thesis show that the *C. burnetii* antibody positivity is highly prevalent in Danish dairy herds and dairy cows, and the prevalence is relatively low in cattle raised for meat production and sent to slaughter. It was also shown that several management and biosecurity factors were strongly associated with antibody positivity in dairy herds and dairy cows. Animal level factors like breed, age and parity were associated with *C. burnetii* antibody positivity in Danish cattle. However, studies show a long persistence within herd seroprevalence in dairy herds.



## SAMMENDRAG

*Coxiella burnetii*, som er årsag til Q-feber hos mennesker og dyr, er en obligat intracellulær bakterie med zoonotisk potentiale. Bakterien forekommer over hele verden og kan inficere en lang række værter, herunder domesticerede drøvtyggere. Ligesom i mange andre europæiske lande er forekomsten af *C. burnetii* blevet rapporteret i flere år i Danmark, og påvisning af agens i dyr i Danmark blev gjort anmeldeligt fra december 2004. Siden den seneste *C. burnetii* epidemi i geder, får og mennesker i Holland i perioden 2007-2010, har epidemiologisk forskning vedrørende Q-feber i både dyr og mennesker fået mere opmærksomhed fra forskerne i mange europæiske lande, herunder Danmark. Dengang var der kun begrænset viden om prævalens, risikofaktorer og dynamik af infektion med *C. burnetii*, og om gyldigheden af de diagnostiske tests, der anvendes til påvisning af infektion eller antistoffer i kvæg. De overordnede mål for denne afhandling var derfor at undersøge epidemiologien af *C. burnetii* antistof positivitet i den danske kvægbestand relateret til frekvens og risikofaktorer, og også at evaluere resultaterne af de diagnostiske tests, der anvendes til at påvise *C. burnetii* antistof positive dyr eller besætninger.

For at nå disse mål er afhandlingen baseret på en række danske epidemiologiske undersøgelser med indsamling af data i 2008-2009 fra 100 tilfældigt udvalgte malkekvægsbesætninger og i løbet af 2012-2013 fra 120 tilfældigt udvalgte malkekvægsbesætninger og i 2012 fra 800 slagtede kvæg. De indsamlede data omfatter laboratorieundersøgelser for tilstedeværelse af *C. burnetii*-antistoffer i mælke- og blodprøver fra individuelle kreaturer og i prøver fra tankmælk i malkekvægsbesætninger. Disse data blev suppleret med data på besætningsniveau fra spørgeskemainterviews med landmænd og med relevante sekundære data på individ- og besætningsniveau fra Den Danske Kvægdatabase.

Et tværsnitsstudie med 120 tilfældigt udvalgte danske malkekvægsbesætninger i juli 2012 viste, at tankmælksprøver af 79 % besætninger var *C. burnetii*-antistofpositive og denne udbredelse var væsentligt højere end ved en tidligere undersøgelse i 2008 (kapitel 3). En negativ indvirkning af stigende besætningsstørrelse og den gennemsnitlige kvægtæthed på risikoen for seropositivitet blev identificeret i samme undersøgelse.

For at identificere risikofaktorer for *C. burnetii*-antistof positivitet på besætningsniveau i danske malkekvægsbesætninger blev tankmælksprøver fra 100 tilfældigt udvalgte besætninger analyseret i en tværsnitsundersøgelse (kapitel 4a). For at få oplysninger om pasningsforhold og smittebeskyttelsesrutiner blev driftslederne telefoninterviewet om arbejdskraft, smittebeskyttelse, opstaldning og besætningssundhed i de 12 måneder forud for undersøgelsen. Resultaterne viste, at deling af maskiner mellem flere bedrifter (Ja versus Nej, odds ratio (OR) = 3,6), besætningskontakt med fremmede mennesker (Ja versus Nej, OR = 4,2), insemination gennemført af andre personer end inseminører (Ja versus Nej, OR = 7,7), om der var en sundhedsrådgivningsaftale med dyrlægen (Ja versus Nej, OR = 4,3), og om dyrlægen tog hygiejniske forholdsregler inden indgang i besætningen (Nej versus Ja, OR = 5) var signifikant associerede med antistof positivitet. Denne undersøgelse bekræfter, at strenge smittebeskyttelsesprocedurer er vigtige for forebyggelse af infektion med *C. burnetii* i malkekvægsbesætninger.



Diagnostiske resultater fra de samme 24 tilfældigt udvalgte malkekvægsbesætninger fra 2008 blev, med supplerende data på ko- og besætningsniveau fra Den Danske Kvæg Database og data fra en spørgeskemaundersøgelse, brugt til at identificere risikofaktorer for *C. burnetii*-antistof positivitet i malkekøerne (kapitel 4b). Resultaterne fra multivariable logistiske regressionsanalyser med hierarkiske strukturer (ko og besætning) viste, at risikoen for at en ko bliver seropositiv var højere i dansk Holstein Frisisk race end i andre malkeracer og om sommeren i forhold til andre årstider. Risikoen øges med stigende paritet og stigende proteinindhold i mælken, men faldt med stigende mælkeydelse og højt fedtindhold i mælken. Besætningsstørrelse, bindestaldssystem, karantæne af nyindkøbte dyr og dyrlægers gode hygiejniske forholdsregler inden de kommer ind i stalden var også signifikant associeret med reducerede odds for *C. burnetii* antistof positivitet.

Prævalensen af antistof positivitet inden for besætningen blev beregnet i 24 stratificeret tilfældigt udvalgte malkekvægsbesætninger (kapitel 4b). Disse besætninger omfattede ti tilfældigt udvalgte besætninger blandt besætninger, der var testet positive i tankmælken ved starten af undersøgelsen, ti tilfældigt udvalgte malkekvægsbesætninger blandt besætninger, der var testet negative i tankmælken, og fire tilfældigt udvalgte malkekvægsbesætninger blandt besætninger, der var test-intermediære i tankmælken (kapitel 4b). Resultaterne viste, at prævalens inden for tankmælkspositive, -intermediære og -negative besætninger var henholdsvis 27 %, 23 % og 0,9 %. Prævalensen inden for tankmælkspositive og -intermediære besætninger forblev stabil ved tre prøveudtagningstidpunkter i en 11-måneders opfølgningsperiode. Dette indikerede en langvarig stabilitet i besætningsprævalensen. Prævalensen steg markant i tankmælksnegative besætninger i samme undersøgelsesperiode. Disse ændringer i prævalensen af testpositive køer i besætningen (kapitel 4b) antydede, at antistof-status i nogle køer ændredes fra positiv til negativ eller omvendt i løbet af undersøgelsesperioden.

Blod-og mælkeprøver fra 568 lakterende køer fra 17 danske malkekvægsbesætninger indsamlet i 2008 blev ved hjælp af latent-klasse-modeller i Bayesiansk analyse (kapitel 5) anvendt til at beregne Se og Sp af den diagnostiske ELISA metode, der blev brugt til påvisning af *C. burnetii* antistoffer i mælke- og blodprøver. Baseret på estimater for differential positive rate (DPR) blev den bedste kombination af Se og Sp fundet ved et cutoff i S/P ratio på 40 for både blod- og mælkeprøver. Ved dette cutoff var Se og Sp i mælk hhv. 0,86 og 0,99, hvorimod Se og Sp i blod hhv. var 0,84 og 0,99. Forskellen mellem Se-estimer for de to tests var ikke statistisk signifikant. Derfor kan valget af testen baseres på rammerne af undersøgelsen. For serologisk overvågning af lakterende malkekøer vil mælke-ELISA-testen være den foretrukne metode, og blod-ELISA-testen kan benyttes til serologiske undersøgelser i ikke-lakterende kvæg. Der blev ikke observeret nogen betinget afhængighed mellem Sp-estimerne for de to testmetoder og Se-estimerne for begge prøver blev kun signifikant reduceret, når betingede kovarianser  $\geq 40$  blev anvendt. Det betyder, at der ikke er nogen absolut afhængighed mellem disse to testmetoder, som påvirker Se og Sp estimaterne. Se og Sp for blod-ELISA beregnet i undersøgelsen blev brugt til at beregne de sande prævalenser for *C. burnetii* seropositivitet i danske slagtedy.

Et andet tværsnitstudie af slagtet kvæg blev gennemført for at vurdere seroprævalensen mod *C. burnetii* i kvæg, der opdrættes til kødproduktion (kapitel 6).

Niveauet af antistoffer blev analyseret i blodprøver fra 800 tilfældigt udvalgte slagte kreaturer fra seks store danske slagterier mellem august og oktober 2012. Bayesianske modeller, med justering for den diagnostiske tests følsomhed, sensitivitet (Se) og specificitet (Sp), blev anvendt til at estimere tilsyneladende prævalens (AP), sand prævalens og sand prævalens med justering for en tilfældig effekt for forskellige racer (kapitel 6). Den samlede AP og TP i danske slagtekvæg var 5,6 %. Signifikante forskelle i forekomsten blev observeret mellem slagtet kvæg af malkeracer (AP = 9,1%, TP = 9,5%) og kødkvægracer (AP = 4,3%; TP = 3,5%), mellem handyr (AP = 3,6%; TP = 2,6%) og hundyr (AP = 9,1%, TP = 9,4%) og mellem typen af oprindelsesbesætning, dvs. malkekvægsbesætninger (AP = 15,1%; TP = 16,7%) versus kødkvægsbesætninger (AP = 4,5%; TP = 3,6%). Resultaterne viste, at kvæg sendt til slagtning har lavere seroprævalens end lakterende malkekøer og blandt de slagtede kreaturer var størstedelen af de test positive dyr af malkerace. En Bayesiansk logistisk regressionsmodel identificeret en positiv sammenhæng mellem *C. burnetii* seropositivitet og alder, race, og antallet af flytninger mellem besætninger inden dyret blev sendt til slagtning.

Som konklusion viser resultaterne af denne PhD-afhandling, at *C. burnetii* antistof positivitet er meget udbredt i danske malkekvægsbesætninger og i malkekøer, og forekomsten er relativt lav i kødkvæg. Det blev også vist, at flere pasnings- og smittebeskyttelsesforhold var stærkt associeret med antistof positivitet i malkekvægsbesætninger og malkekøer. Individfaktorer som race, alder og paritet var forbundet med *C. burnetii*-antistof positivitet i dansk kvæg. Det blev også fundet, at *C. burnetii* antistofstatus i enkeltdyr ikke var stabilt i en længere periode i malkekøer i Danmark. Derimod viser vores undersøgelser at seroprævalensen inden for besætningerne er ret konstant.



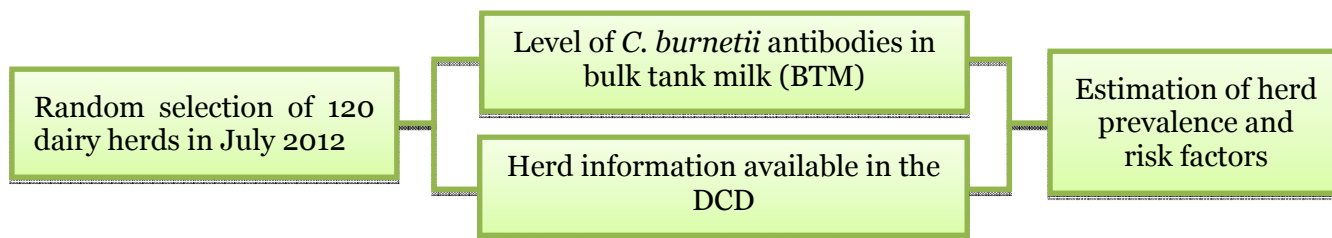
## OUTLINE OF THE THESIS

This PhD thesis is organized into eight chapters -

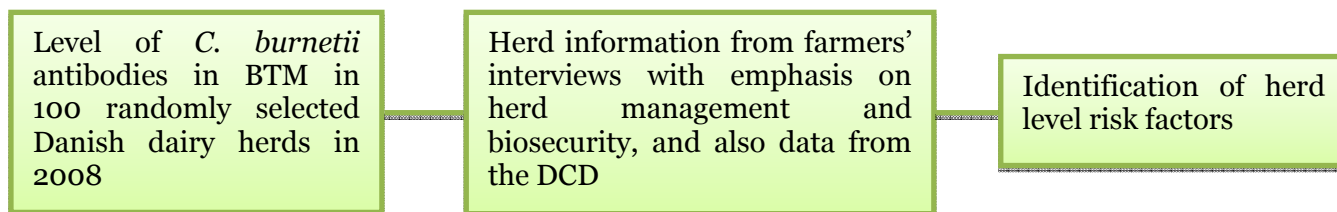
The first chapter (Chapter 1) provides a short introduction of the background and the overall aim of this thesis.

Chapter 2 contains a general overview of Q fever/*C. burnetii* infection, including bacteriology, pathogenesis, distribution, transmission, reservoir, risk factors, clinical manifestation in animals and humans, diagnosis, treatment and prevention and control.

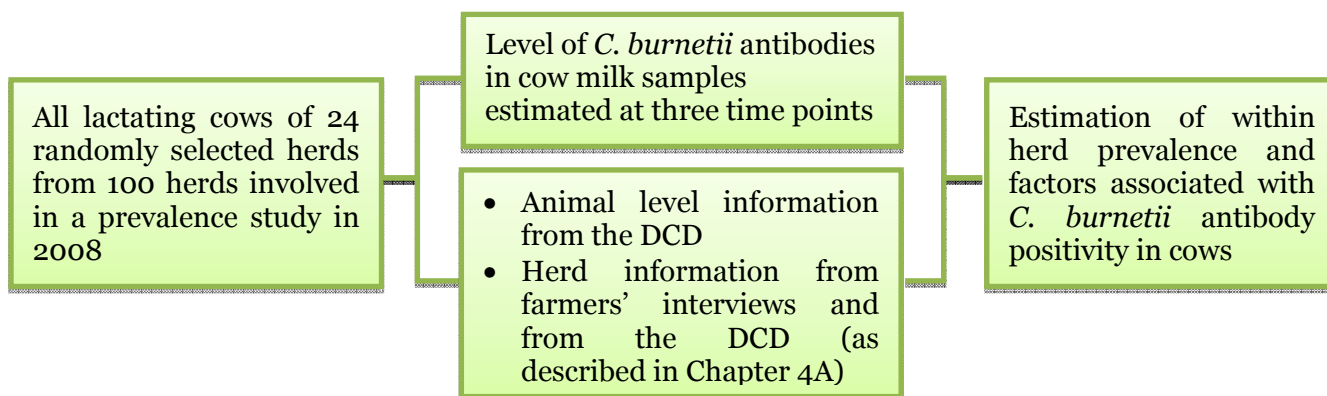
Chapter 3 describes the prevalence of *C. burnetii* antibody positivity in Danish dairy cattle herds. This chapter also briefly describes the risk factors associated with seroprevalence in dairy cattle herds. Only those risk factors, which were readily available in the Danish Cattle Database (DCD), were included in this study. The study was designed as follows:



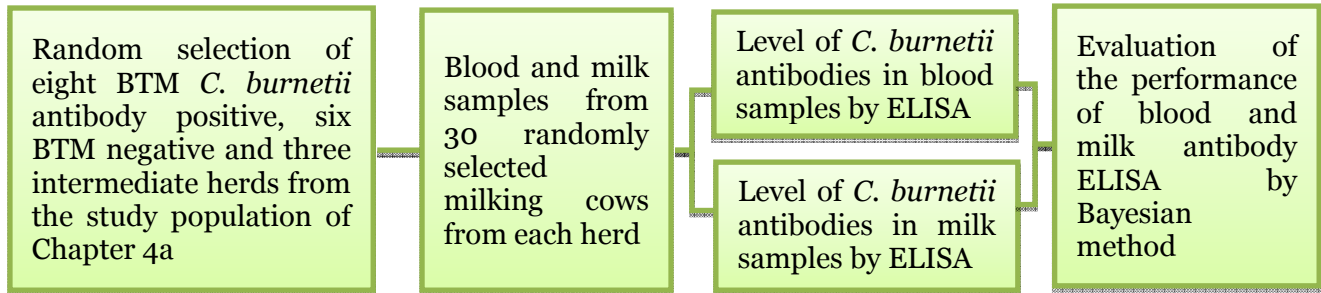
Herd level and cow level risk factors associated with *C. burnetii* antibody positivity in Danish dairy cattle are discussed in Chapters 4A and 4B. Chapter 4A includes a herd level risk factor study designed as follows:



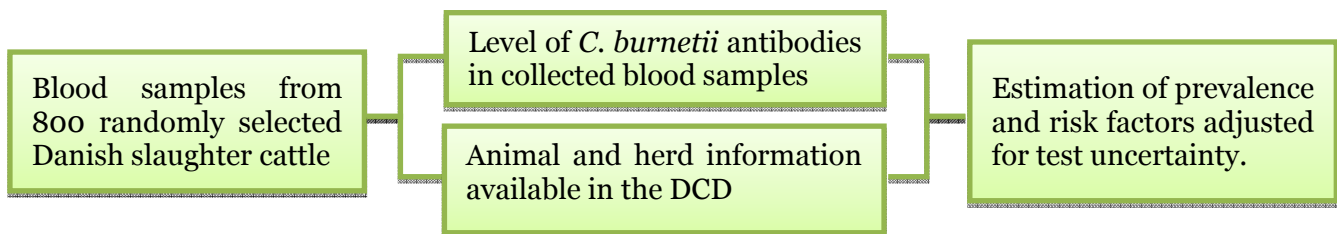
Chapter 4B describes factors associated with *C. burnetii* antibody positivity in Danish dairy cows, and includes lactating cows from 24 randomly selected dairy herds from the 100 herds described in Chapter 4A. The design and data structure of this study are as follows:



Chapter 5 evaluates the performance of the antibody ELISA used for identifying *C. burnetii* antibody positive cows in a study design as follows:



Chapter 6 describes the prevalence and risk factors of *C. burnetii* antibody positivity in Danish slaughter cattle. Estimates of this chapter were adjusted from uncertainty of diagnostic tests. Test characteristics obtained from Chapter 5 were used to calculate true prevalence and risk factors in this chapter. The design and data structure of this study are:



A general discussion of the results obtained from the different studies, perspectives and conclusions for future research and practical applications are presented in Chapter 7.

Chapter 8 of this thesis includes the annexes.

# **CHAPTER 1**

## **GENERAL INTRODUCTION**

### **SYNOPSIS**

This chapter provides a brief introduction on the magnitude and distribution of *Coxiella burnetii* infection (Q fever) in humans and cattle in the European Union; a brief description of cattle production in Denmark and the epidemiological studies on Q fever in cattle in Denmark. This chapter also presents the objectives and related research questions of the thesis.



# GENERAL INTRODUCTION

## *Coxiella burnetii* infection (Q fever) in the European Union (EU)

### Occurrence in humans

*Coxiella burnetii*, a highly contagious bacterium, is the causative pathogen of Q fever. Q fever is a well-known zoonotic disease with worldwide distribution, and is enlisted in the multiple species disease category on the OIE (World Organization for Animal Health) list. It is one of the 47 enlisted communicable diseases within the EU legal framework of communicable disease surveillance and notification (Commission Decision 2000/96/EC). According to this legal framework, human Q fever cases must be notified in EU member states (MS) using a harmonised case definition (EFSA, 2010). Generally, Q fever occurs in almost all EU MSs, but persists at a low frequency. Reported human cases are either sporadic or discrete clusters from a specific source (Orr et al., 2006; Wilson et al., 2010), or a part of clusters of known community based outbreaks. The annual proportion of notification ranges from 500 to 2000 cases per annum (EFSA, 2010). Human outbreaks in the EU are infrequent and typically 1-3 human outbreaks are reported annually. Outbreaks are relatively small involving 10 to 500 human cases, and generally do not persist constantly over the years in the same country (EFSA, 2010).

In 2007, 637 confirmed human cases were reported from 14 MSs, of which the majority of cases were reported from the Netherlands (1.03 cases/10<sup>5</sup>) and Slovenia (4.6 cases/10<sup>5</sup>) (EFSA, 2010). The first human outbreak in the Netherlands was reported in 2007, from a localised area which was believed to be linked with an outbreak on a goat farm (Roest et al., 2011). In subsequent years, the Netherlands faced several human outbreaks of unprecedented size within the same cluster, however, with wider geographical spread (Karagiannis et al., 2007; Schimmer et al., 2009; Schimmer et al., 2008; Schimmer et al., 2010). In 2008, 24 EU MSs reported 1594 confirmed human cases (0.5 cases/10<sup>5</sup>), which was 170% higher than 2007 (EFSA, 2010). This sharp increase was mainly due to the increase in the Netherlands (1011 cases; 6.2 cases/10<sup>5</sup>) and Germany (370 cases; 0.5 cases/10<sup>5</sup>). The total number of confirmed human cases in EU MSs increased further (1988 cases from 15 MSs) in 2009, and yet again the majority of cases were attributed to the Netherlands (1623 cases) and Germany (191 cases) (EFSA, 2012; EFSA/ECDC, 2010). Twenty four MSs reported a total of 1414 confirmed human cases (0.36cases/10<sup>5</sup>) in 2010, of which seven MSs reported zero cases. Eighty-one percent of the total reported cases occurred in the Netherlands and Germany (EFSA, 2012). France reported human cases of Q fever for the first time in 2010 (286 cases; 0.44 case/10<sup>5</sup>). This proportion of notifications in all MSs was 28.9% less than in 2009, and the largest decrease was observed in the Netherlands (67%) (EFSA, 2012). Massive veterinary control measures were put in place, i.e. culling all pregnant small ruminants from Q fever positive farms, a ban on breeding and transport and vaccination, these precautions were likely the reason for the reduction of human Q fever cases in the Netherlands. However, the adverse weather conditions in 2010, might have also contributed to this reduction (Roset et al. 2011). Descriptive analysis of confirmed cases from 2007 to 2010, shows that the proportion of notifications was higher in the age group 45-64, with the highest proportion (1.09/10<sup>5</sup>) in 2009 (EFSA, 2010; EFSA, 2012; EFSA/ECDC, 2009; EFSA/ECDC, 2010).



Cases were rarely reported among children less than 15 years of age. A proportion of notifications of confirmed cases was higher in men than in women, with male to female ratios of 1.78 and 1.58 in the year 2007 and 2009, respectively (EFSA, 2010). Seasonal variation was observed in notification proportions, and most outbreaks were reported in May to July with few exceptions (EFSA, 2010; EFSA, 2012). A large outbreak was observed in August 2009 in the Netherlands; and in 2010, another sharp pick of reported cases was observed, which was probably related to lambing or kidding season (EFSA, 2012). Among the EU MSs, Austria and Denmark do not have any national active surveillance system for human Q fever; i.e. only passive surveillance. These two MSs along with Italy have not reported any human cases so far (EFSA, 2010; EFSA, 2012).

The Q fever surveillance system in the EU is based on the notification of clinical cases, but no harmonised surveillance system is available to assess the seroprevalence (EFSA, 2012). Several serological studies were conducted to assess the rate of seroconversion. Comparison of the outputs of these studies is not always possible, as the studies varied significantly in terms of design, target population and diagnostic tests used. However, broadly between 2-10% of the total population included in these studies were exposed to *C. burnetii* infection (EFSA, 2010; EFSA, 2012; EFSA/ECDC, 2010). In recent studies, a high level of seroconversion was observed in occupational groups or in groups with extended exposure to animals (Bartolome et al., 2007; Bosnjak et al., 2010; Cisak et al., 2003; Monno et al., 2009; Orr et al., 2006). But, there is no scientific evidence that the occurrence of the clinical disease was higher in these risk groups. A recent study in Denmark involving risk group populations (i.e., farmers, farm workers, veterinarians, inseminators, etc.) indicated that the risk of seroconversion is higher in veterinarians than in other occupational groups (Bosnjak et al., 2010). Although serological studies varied substantially, results indicate that clinical cases of Q fever were significantly underreported. This is because of the pleomorphic nature of the disease (mostly asymptomatic or with non-specific symptoms), and failure to identify the infection in animal hosts. Thus, estimation of the real number of confirmed human Q fever cases in the EU is troublesome (EFSA/ECDC, 2009).

## **Occurrence in cattle**

Q fever/*C. burnetii* infection is included in list B of diseases (*‘other zoonoses and zoonotic agents that shall be monitored according to the epidemiological situation in an MS*) in Annex I of Directive 2003/99/EC. But, it is not included in the notifiable disease list in domestic ruminants in the EU legislation (Directive 82/849/EEC), or in the Regulation 2160/2003/EC for zoonosis control options. Therefore, there are no rules for notification or monitoring/surveillance and also for control of intra-country imports concerning Q fever. This disease is nationally notifiable in only 14 EU MSs (Sidi-Boumedine et al. 2010). According to veterinary legislation (Act no. 432 of 09/06/2004), Q fever has been a notifiable disease in Denmark since December 2004 (Anonymous, 2010). Based on the available information in the EFSA/ECDC zoonoses database, it is not possible to make a conclusive statement about the exact scenario of Q fever in domestic ruminants in EU MSs. This is because disease reporting systems in EU MSs are not harmonised, and significant variation is observed in the level of reporting. Moreover, most of these reports were based on clinical investigations/suspected samples (EFSA, 2010; EFSA, 2012). So, animals included in these investigations do not represent the population.

This indicates the need for a harmonised monitoring/surveillance system in the EU to make an unbiased comparison of Q fever occurrence in animals between EU MSs and over time. A scientific report on developing harmonised schemes for monitoring and reporting of Q fever in animals in the EU was submitted to the EFSA in 2010 (Sidi-Boumedine et al., 2010).

From 2008 to 2010, 18 EU MSs reported information regarding Q fever/*C. burnetii* infection in domestic ruminants in the ECDC/EFSA zoonoses database, of which eight MSs reported infection/disease in cattle (EFSA, 2012; EFSA/ECDC, 2010). From the available information, it is obvious that infection with *C. burnetii* is endemic in all of these reporting countries. Although there are variations in prevalence among the MSs, no spatial cluster was observed across Europe, and no upward trend of Q fever was observed within EU MSs (EFSA, 2010). However, caution should be taken in interpreting this information, as the reporting system in the EU is not harmonised. In 2010, the occurrence of *C. burnetii* infection in cattle at the animal level was 2.8% (EFSA, 2012), which was lower than that in 2008 (9.9%) and 2009 (9.0%) (EFSA, 2010). Denmark reported the highest occurrence in bovines (29%), followed by Spain (11.6%). Reports from both countries were based on suspected samples and serology, which may have contributed to the comparatively higher occurrence. Denmark reported the largest decrease in occurrence of *C. burnetii* in cattle in 2010, compared to the occurrence in 2009 (54.5%). In MSs, where more than 2000 cattle were sampled, the proportion of positivity ranged from 0.4% to 10.5%. Only four MSs reported herd level information regarding *C. burnetii* infection in cattle in 2010 (EFSA, 2012). The proportions of herd level positivity were reported to be 25.5%, 11.9% and 6.1% in 2008, 2009 and 2010, respectively (EFSA, 2010; EFSA, 2012). Yet again, Denmark reported the highest herd-level occurrence (75%) based on serology and non-randomly selected clinically suspected samples; followed by Sweden (61.4%). The report from Sweden also based on serology and sampling, was carried out in a known high prevalence area. Germany and Poland examined a substantial amount of herd-level samples and reported a 17.7% and a 0.6% occurrence, respectively.

On top of this official reporting system, several prevalence studies of *C. burnetii* infection in cattle were carried out in EU MSs and non-EU countries, and most of them were seroprevalence studies. The results of these studies were summarised in a review article (Guatteo et al., 2011). However, studies varied greatly in terms of study design, sampling population and diagnostic tests used, and only in few studies were animals/herds selected randomly. The animal level prevalence ranged from 6.2% to 22% in the studies where animals were selected by random sampling (Cabassi et al., 2006; Capuano et al., 2001; McCaughey et al., 2010; Ruiz-Fons et al., 2010). The highest prevalence was reported in Italy (Cabassi et al., 2006). Prevalence is generally higher in dairy cattle than beef cattle (McCaughey et al., 2010; Ruiz-Fons et al., 2010; Ryan et al., 2011). The herd level prevalence ranged from 29% to 71.2% in the studies where herds were selected randomly (Agger et al., 2010; Czaplicki et al., 2012; McCaughey et al., 2010; Ruiz-Fons et al., 2010). The herd level prevalence in Denmark was 59% in 2008 (Agger et al., 2010), which was higher than what was stated in the EFSA report (46.4%) in the same year (EFSA, 2010).

## Cattle production in Denmark

Cattle production in Denmark consisted of 13,387 cattle herds in 2011, of which 4,062 and 9,325 herds were dairy and non-dairy (with suckler cows + without dairy cows) herds, respectively (Anonymous, 2012a; Anonymous, 2012b). In 2011, a 4.41% reduction in the number of dairy herds was observed, compared to 2010. The average herd size of the total cattle population was 117.1 heads/herd in 2011, with a visible difference between dairy and non-dairy herds (Anonymous, 2012a; Anonymous, 2012b). In 2011, the herd size of dairy cattle (132 heads/herd) increased about two fold, compared to 2001 (Anonymous, 2012b). Sixty-one percent of dairy herds contained more than 100 heads of animals and represented 84.4% of the total dairy cattle population (Anonymous, 2012a). In contrast, the average herd size of the suckler cows in 2011 (11.9 head/herd) remained the same as in 2009 and 2010. Sixty-two percent of total suckler cow herds contained less than ten cows and represented only 23.1% of the total population (Anonymous, 2012a). Most of the cattle herds were in Jutland, with the highest herd and cattle proportion in the southern part of Jutland. According to 2011 statistics, 24.6% of the total cattle herds were located in this part of the country, which represented 32.5 % of the total cattle population (Anonymous, 2012a).

The total number of cattle was estimated to be 1,615,000 in 2011, of which 579,000 were dairy cattle (Anonymous, 2012a; Anonymous, 2012b). Danish Holstein is the predominant breed among dairy cows (72.86%), followed by Danish Jersey (12.55%) and crossbred cows (8.36%). Average milk yield per cow in October year 2010/11 was 8,919 kg which was higher than the EU average (EUROSTAT, 2012). In 2011, 21.9% of total export values of agricultural products were gained from dairy products in Denmark (Anonymous, 2012b). The annual average per capita milk and milk products (excluding cheese) consumption in Denmark was recorded 137.4 kg in 2008 (Anonymous, 2009). Danish beef cattle population comprises of several breeds, of which Limousine and Hereford are predominant (Anonymous, 2009). In 2011, a total of 513,134 cattle were slaughtered in the authorized abattoirs in Denmark which produced approximately 133,000 tonnes of beef (Anonymous, 2012a). Denmark was the country with the second highest consumption of beef in the EU, with an annual average per capita consumption of 28.1 kg (Anonymous, 2012a). According to Eurostat estimations in 2011, the annual average per capita in the EU countries was 16.7 kg with the highest consumption in Luxemburg (EUROSTAT, 2012). Beef and veal shares the 32.7% of total meat consumption in Denmark (Anonymous, 2012a).

## Epidemiological studies on Q fever in cattle in Denmark

Although notifiable, no national active surveillance system exists for Q fever/*C. burnetii* infection in domestic animals in Denmark (Anonymous, 2010) except for the classical passive surveillance where animal owners have the obligation to call their veterinary practitioner when animals show clinical signs of a severe disease. Herd level seroprevalence of *C. burnetii* infection in cattle was reported in a few epidemiological studies. Proportions of positivity were 22.2%, 37.5% and 49.6% in 2003, 2004 and 2006, respectively (Christoffersen, 2007). In 2007, the proportion of positive herds was 57% (Bødker and Christoffersen, 2008). However, the above mentioned studies were based on

non-randomly selected clinically suspected samples, or samples submitted by veterinarians for routine testing of animals intended for export. Therefore, these samples were not representative of the total population, and the results of these studies could not be generalised.

In 2008, bulk tank milk (BTM) samples of 100 randomly selected dairy herds were tested for the presence of *C. burnetii* antibodies in a prevalence study; of which, 59 % of herds were BTM positive (antibody level [S/P]  $\geq 40$ ) (Agger et al., 2010). Thirty percent of these 100 samples were negative (S/P < 30), and the remaining 11% were intermediate (S/P 30 -39). Ten positive, ten negative and four intermediate herds; and animals within these herds from the previous study were followed up on in a number of studies. Nielsen et al. (2011) did not find any association between stillbirth and/or neonatal mortality with the level and monthly changes of BTM antibodies in these 24 herds. In another study, cow milk samples from 12 herds (ten positive, one negative and one intermediate) were collected repeatedly at three time points over an 11-month period, and tested by ELISA and real time PCR (Angen et al., 2011). Results showed that the level of bacterial shedding and antibodies in cow milk were stable in most of the herds; and bacterial shedding was significantly associated with the antibody level in cow milk or in BTM. The level of bacterial shedding increased with increasing parity and increasing protein concentration. The relationship between the presence and level of *C. burnetii* infection and cotyledonary lesions were studied by Hansen et al. (2011). In this study, 170 cotyledons from 19 herds were analysed by real time PCR, 110 of which were examined by histology, immunohistochemistry and fluorescence in situ hybridisation (in case of high infection level only). Placental infection was found in cows from herds at all BTM antibody levels, with higher levels in positive and intermediate herds. Mild cotyledonary and no inflammatory changes were observed for *C. burnetii* infection. Beyond identification of bacteria and estimation of herd seroprevalence, these previous studies barely tried to estimate the animal level prevalence and herd and animal level risk factors of both dairy and beef cattle. Therefore, the important epidemiological indices such as frequency, distribution and risk factors of *C. burnetii* infection in the Danish cattle population remain unknown. Furthermore, the performances of the diagnostic tests used in these previous studies were not thoroughly evaluated under Danish conditions, and therefore, the validity of the findings of these previous studies may be under question. These points explain the necessity for further epidemiological research on Q fever in the Danish cattle population.

## Aim of the thesis

The overall objective of this thesis was to investigate the epidemiology of the *C. burnetii* antibody positivity in the Danish cattle population. The main focus was on the estimation of prevalence and risk factors of *C. burnetii* antibody positivity in Danish cattle; and also on evaluation of the performance of the diagnostic tests used in identifying a positive *C. burnetii* antibody in animals or herds. To ascertain this objective, data was collected from Danish cattle under a series of epidemiological studies with several specific objectives:

**Objective 1:** To estimate the prevalence of *C. burnetii* antibody positivity in Danish dairy cattle herds. The research question addressed under objective 1 is:

- a) Was the prevalence of *C. burnetii* antibody positivity in Danish dairy cattle herds similar in 2008 and 2012?

**Objective 2:** To investigate the risk factors associated with *C. burnetii* antibody positivity in Danish cattle. The research questions addressed under objective 2 are:

- a) What are the risk factors for Danish dairy cattle herds found to have positive *C. burnetii* antibodies?
- b) What are the cow-level and herd-level risk factors associated with *C. burnetii* antibody positivity in Danish dairy cows?

**Objective 3:** To estimate the sensitivity and specificity of the antibody ELISA test used for detection of *C. burnetii* antibodies in milk and blood samples from Danish dairy cows. The research question addressed under objective 3 is:

- a) Do the sensitivity and specificity estimates of this diagnostic test differ when applied in the analysis of milk and blood samples of dairy cows?

**Objective 4:** To estimate the prevalence and risk factors of *C. burnetii* antibody positivity in Danish slaughter cattle adjusted for diagnostic test uncertainty. The research questions addressed under objective 4 are:

- a) What is the true prevalence of *C. burnetii* antibody positivity in Danish slaughter cattle?
- b) What are the risk factors for the true prevalence of *C. burnetii* antibodies in Danish slaughter cattle?

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## **CHAPTER 2**

# ***COXIELLA BURNETII* INFECTION (Q FEVER): AN OVERVIEW**

### **SYNOPSIS**

This chapter presents a brief overview of *Coxiella burnetii* infection (Q fever), which includes bacteriology, pathogenesis, epidemiology (distribution, transmission, reservoir and risk factors), clinical manifestation and diagnosis of infection and disease, treatment, general prevention and control measures; and finally, some concluding remarks.



# **COXIELLA BURNETII INFECTION (Q FEVER): AN OVERVIEW**

## **Bacteriology**

*C. burnetii* is a non-motile obligate intracellular bacterium. This pleomorphic rod shaped organism possesses a cell membrane similar to that of other Gram-negative bacteria (Maurin and Raoult, 1999). Although Gram-negative, it cannot be stained by the Gram staining method, but Gimenez's method is, however, useful (Gimenez, 1965). Stamp, Macchiavello, modified Ziehl–Neelsen method and modified Koster, can also be used to stain bacteria. *C. burnetii* was classified as a member of the order *Rickettsiales*, family *Rickettsiaceae*, the tribe *Rickettsiae* and the genera *Rickettsia*. The bacterium was reclassified into the order *Rickettsiales*, based on 16S rRNA sequence analysis and phylogenetic studies (Maurin and Raoult, 1999). It now belongs to the gamma subdivision of the *Proteobacteria*. Within this *Proteobacteria* group, *Francisella* and *Legionella* are the phylogenetic neighbours of *C. burnetii* (Maurin and Raoult, 1999). After internalisation, *C. burnetii*, like *Ehrlichiae*, remains within a parasitophorous vacuole (PV) of infected cells and completes all of its life cycle stages within this vacuole (McCaul and Williams, 1981). A high number of bacteria are generated within the PV, as they replicate at an estimated doubling time of 20–45 h (Mertens and Samuel, 2007). Electron microscopy shows that the organism has two metabolic forms: 1) the small-cell variant (SCV) is a compact, small rod and has a very electron-dense centre of condensed nucleoid filament; and 2) the large-cell variant (LCV) which is comparatively larger and less electron-dense than the metabolically active intracellular form of *C. burnetii*. Unlike other members of the tribe *Rickettsiae*, *C. burnetii* is highly resistant to physical and chemical exposure, and it can form a small structure similar to an endospore within the infected cell (McCaul and Williams, 1981). The organism is also able to resist extreme environmental conditions such as desiccation, ultraviolet light and survives in the environment for a long time (Babudieri, 1959; Scott and Williams, 1990). The antigenic variation (phase variation) of the bacterium is determined by lipopolysaccharides (LPS) (Angelakis and Raoult, 2010). Phase I bacteria (wild virulent type) with a smooth full length LPS were isolated from infected humans, animals and arthropods (Maurin and Raoult, 1999). Phase I bacterium converts to an avirulent phase II with rough LPS after several passages in embryonated egg or cell cultures (Hotta et al., 2002). However, virulent phase II, an intermediate phase with semi-rough LPS, has been described in some of the studies (Amano et al., 1987; Vodkin and Williams, 1986). Based on the restriction fragment length polymorphism (RFLP), strains of *C. burnetii* are grouped into six (I–VI) genomic groups (Maurin and Raoult, 1999). The virulence and the pathogenicity of the *C. burnetii* are associated with genetic characteristics, plasmid groups and type of strains (Hendrix et al., 1991), and also with host factors such as pregnancy (Woldehiwet, 2004). Plasmid groups I, II and III are responsible for acute infection, whereas groups IV and V are the cause of chronic infection (Hendrix et al., 1991).

## Pathogenesis

The pathogenesis of *C. burnetii* infection in humans and animals is not clearly understood. But, it is believed that bacterial LPSs play an important role in the pathogenesis of Q fever in both humans and animals (Angelakis and Raoult, 2010, Maurin and Raoult, 1999). The organism probably follows the oropharyngeal route as its port of entry into the lungs and intestines of both humans and animals (Gardon et al., 2001; Woldehiwet, 2004). It is highly infectious, and a very low dose is sufficient to initiate infection (McQuiston et al., 2002). Primary multiplication takes place in the regional lymph nodes after the initial entry, and a transient bacteraemia develops which persists for five to seven days, as shown in sheep (Woldehiwet, 2004). An old study has claimed that the bacteria localise in the mammary glands or in the placenta of pregnant animals after the initial multiplication (Babudieri, 1959). *C. burnetii* enters monocytes or macrophages; the only known target cells, by phagocytosis in humans (Marrie et al., 1996; Mege et al., 1997). The phagocytotic process differs for phase I and phase II bacteria. Phase II bacteria enter the host's cells through CR3-receptor mediated phagocytosis by activating the CR3 receptors (Mege et al., 1997). On the other hand, the attachment of phase I *C. burnetii* to a monocyte is aided by leukocyte response integrin (LRI)  $\alpha v \beta 3$ , and integrin-associated protein (IAP) (Mege et al., 1997). In spontaneous infections, the phase I *C. burnetii* survives within the phagocytic cells, as the internalisation of the bacteria by these cells is poor. In contrast, uptake of the phase II *C. burnetii* by monocyte is rapid (Mege et al., 1997). After internalisation in phase II, *C. burnetii* initiates phagolysosomal pathways within monocytes which rapidly kill the bacteria (Maurin and Raoult, 1999).

Following passive entry, invading *C. burnetii* bacteria embed in the phagosomes of host cells. Infected phagosomes fuse progressively with lysosomes to form a large vacuole, PV (Hackstadt and Williams, 1981; Howe et al., 2003). *C. burnetii* is an acidophilic bacterium, and therefore, can survive and multiply in an acidic environment within the PV. Acidic pH allows the entry of necessary nutrients for bacterial metabolism (Chen et al., 1990; Hackstadt and Williams, 1983; Hendrix and Mallavia, 1984). It also alters the activity of antibiotics and prevents bacterial killing (Hackstadt and Williams, 1981). Within the PV, *C. burnetii* undergoes a complex intracellular life cycle to form a spore-like stage. SCVs, the extracellular metabolically inactive and highly resistant form of the bacteria, attach to the host's cell membrane. After the formation of PV, the acidic environment triggers the activation of the SCVs to form LCVs. LCVs then undergo a sporulation-like process to form a resistant, spore-like form of the bacterium (McCaul and Williams, 1981). Binary fission is the process of cell division for both activated SCVs and LCVs.

*C. burnetii* is capable of producing chronic infection in both humans and animals (Baca and Paretsky, 1983), which may persist for months and perhaps, even for years (Marmion et al., 2005; Raoult et al., 2000). Although *C. burnetii* persists for a long time within the host cell, it has a very slow intracellular multiplication. Therefore, it may not alter the host cell's characteristics significantly (Maurin and Raoult, 1999). Moreover, the septum positions itself asymmetrically close to one end of the mother LCV cell during the cell division process to develop a sporulation-like form. So, only one daughter cell receives the PV when an infected cell divides (Hechemy et al., 1993). This phenomenon might also play a role in the persistence of the *C. burnetii* infection. *C. burnetii* bacteria show a special affinity to reproductive tissues in pregnant animals. In an experimental study, the bacteria

have been shown to first infect the trophoblastic cells of chorioallantoic membranes in pregnant goats, and maximum bacterial proliferation was observed in the foetal placental part (Sanchez et al., 2006). This feature might explain why *C. burnetii* infection is sometimes associated with abortion.

Both cell-mediated and humoral immunities are required for the clearance of intracellular pathogens (Casadevall and Pirofski, 2006). In the case of *C. burnetii* infection in animals, cell-mediated immune response is the key for bacterial elimination (Turco et al., 1984), and the role of humoral immunity is not clearly understood. Two cytokines, namely interferon  $\gamma$  (INF- $\gamma$ ) and tumour necrosis factor (TNF) play the key role in the *C. burnetii* elimination process (Dellacasagrande et al., 1999; Turco et al., 1984). An in-vitro study has shown that INF- $\gamma$  has the capacity of restricting the growth of *C. burnetii* in infected monocytes (Turco et al., 1984). This cytokine, together with TNF induces an apoptosis to kill the *C. burnetii* infected monocytes (Dellacasagrande et al., 1999; Mege et al., 1997). It is believed that INF- $\gamma$  mediates the killing of *C. burnetii* through the alteration of conditions within the phagosomes of infected cells (Ghigo et al., 2002). Phase I *C. burnetii* stimulates the synthesis of TNF from the infected monocytes in humans (Capo et al., 1996). It is a general belief that antibodies have little impact on *C. burnetii* infection (Norlander, 2000). However, during the bacteraemic phase of acute infection, antibodies can play a role in bacterial elimination. But, in chronic infection, immune complexes may cause pathology (Raoult, 1990). Antibodies are produced following the infection and antigenic properties of invading *C. burnetii* determine the type of antibody production. Infection with phase II *C. burnetii* induces secretion of both IgG and IgM, whereas phase I *C. burnetii* can only induce IgM production (Maurin and Raoult, 1999).

## Distribution

Until the recent outbreaks of Q fever in the Netherlands, *C. burnetii* infections in animals had gained very limited attention, and it was considered to be an infection with very little impact on the health and production of domestic animals. Therefore, information on geographical distribution of animal coxiellosis was very limited. In a recent review article, it was stated that since 1960, *C. burnetii* infection in domestic ruminants has been reported in 35 countries which cover five continents (Guatteo et al., 2011). Human Q fever has been reported from almost all parts of the world, which includes all continents except Antarctica (Marrie, 1990; Woldehiwet, 2004). Since 1956, human Q fever has been reported from over 70 countries (Woldehiwet, 2004). The only country that claims to be free from Q fever is New-Zealand. However, a study found evidence of seropositivity in humans (three positive out of 97 serum sample tested) in New-Zealand (Greenslade et al., 2003). Epidemiological studies in man and animals suggested that *C. burnetii* infection is highly prevalent in tropical regions (Woldehiwet, 2004).

## Transmission

*C. burnetii* bacteria have unique properties which contribute to their transmission between hosts: (1) unlike other members of the *Rickettsiaceae*, the life cycle of *C. burnetii* is not dependent on arthropods as vectors; and (2) the SCV form is highly resistant in external environments. Inhalation of contaminated fomites is the most common mode of

transmission to humans (Marrie, 1990). Domestic ruminants serve as the most important known source of human infection (Maurin and Raoult, 1999). However, many other animal species may play a role in *C. burnetii* transmission. Parturient cattle, ewes and goats can excrete very high quantities of bacteria through amniotic fluid and foetal membranes (Arricau-Bouvery et al., 2005). Direct contamination by aerosols may occur from these products of parturition. Abortive animals may continue to shed bacteria for a long period (Berri et al., 2001). Infected animals may also shed *C. burnetii* in milk, urine, faeces and uterine discharge (Arricau-Bouvery et al., 2005; Guatteo et al., 2006). Milk is the most common shedding route for goats and cattle, whereas ewes shed bacteria most commonly in faeces and vaginal mucus (Rodolakis et al., 2007). Excreted bacteria contaminate fomites such as wool, clothing, straw, manure etc., which may serve as vehicles for transmission (Tissot-Dupont et al., 1992). Indirect transmission to humans may result from the handling of contaminated farm utensils, straw or manure, or by dust from farm vehicles. *C. burnetii* may also spread through the air, and therefore, infection may occur in a person without any history of animal contact (Marrie and Raoult, 1997; Tissot-Dupont et al., 1999). However, in some studies, it was shown that wind spread is not an important mode of *C. burnetii* transmission (Gardon et al., 2001). Ingestion of contaminated milk and milk products could be a potential source of human infection (Babudieri, 1959; Fishbein and Raoult, 1992; Tylewskawierzbanowska et al., 1991). However, it was not evident in an experimental study (Cerf and Condrón, 2006). Rare, but sporadic cases of human-to-human transmission of Q fever have occurred to attendants during autopsies and following contact with a pregnant woman (Harman, 1949; Marmion and Stoker, 1950; Raoult and Stein, 1994). Sexual transmission of *C. burnetii* infection was also reported in a study (Milazzo et al., 2001).

Inhalation of bacteria from the infected environment and ingestion of contaminated straw, hay or pasture are likely the most important sources of *C. burnetii* infection in animals. Animals which live in or come in contact with contaminated premises or infected animals may acquire the infection. Sandford et al. (1994) described three newly purchased goats with a known history of *C. burnetii* infection introducing infection and abortion in a goat farm. Dogs and wild carnivores may be infected by ingestion of contaminated ruminant placenta or birth products, or by the aerosol route (Angelakis and Raoult, 2010). Although ticks are not essential for the life cycle of *C. burnetii*, they may still play an important role in transmission of the infection in wild vertebrates (Lang, 1990). Transmission of *C. burnetii* infection to guinea pigs via tick bites was shown in an experimental study. Possible sexual transmission of *C. burnetii* infection was reported in mice (Kruszewska and Tylewskawierzbanowska, 1993). Insemination might be a source of infection, as viable *C. burnetii* was found in bull semen (Kruszewska and Tylewskawierzbanowska, 1997).

## Reservoir

*C. burnetii* is considered to be a pathogen with no host specificity and it was shown that infection may occur in a wide range of vertebrates, which includes wild and domestic mammals, birds and arthropods (Angelakis and Raoult, 2010, Babudieri, 1959, Maurin and Raoult, 1999). Babudieri (1959) in his review paper stated that *C. burnetii* bacteria were detected in virtually all of the animal kingdom. However, the clinical form of the infection, Q fever, is mostly seen in humans. Cattle, sheep and goats are considered to be the most

common source of human infection (Marrie, 1990). *C. burnetii* infection, without showing any clinical signs has been reported in horses, pigs, dogs, cats, camels, and buffalos, and also from wild and domestic chickens, pigeons, ducks, geese and turkeys (Babudieri, 1959). This organism was also isolated from rabbits, cats, squirrels, mice, deer and many other free living animals ( Marrie et al., 1986). *C. burnetii* was also isolated from 40 species of ticks (Maurin and Raoult, 1999).

## **Risk factors**

### **Agent factors**

The severity of the infection depends on the strains of the infecting bacteria. Phase I type bacteria are more virulent than the phase II type (Amano and Williams, 1984). Acute infection in humans is caused by *C. burnetii* genomic type I-III, whereas type IV and V are responsible for chronic infection. The virulence of type VI is unknown (Hendrix et al., 1991). Clinical manifestations of Q fever are sometimes dependent upon the route of invasion. Pneumonic lesions are often observed when the infection is gained by inhalation. On the other hand, an infection through the oral route may produce hepatitis (Marrie et al., 1996).

### **Host factors**

Age and gender are the two risk factors which are shown to influence the occurrence of Q fever in humans. People aged 30-60 years are the most vulnerable group, and the clinical disease is mostly prevalent in men (Maurin and Raoult, 1999; Tissot-Dupont et al., 1992). However, other studies did not find any relationship between gender and the occurrence of Q fever (Marrie and Pollak, 1995; Tissot-Dupont et al., 1992). People with a previous history of valvulopathy, an immunosuppressive disease like AIDS and pregnant women (Raoult, 1990; Raoult and Marrie, 1995; Raoult and Stein, 1994) are more prone to develop chronic Q fever. People in certain occupations like veterinarians, animal farm workers, abattoir workers and laboratory personnel are at a higher risk of being infected or seropositive than others; and studies show a comparatively higher prevalence in these groups (Bosnjak et al., 2010, Maurin and Raoult, 1999).

A relationship of *C. burnetii* infection with age and sex was also found in animals, particularly in cattle. Several studies have shown that the prevalence of *C. burnetii* infection increases with age or with the number of parity in cattle and sheep (Bottcher et al., 2011; Garcia-Ispuerto et al., 2011; Kennerman et al., 2010; McCaughey et al., 2010). Prevalence is higher in dairy cows than in beef cattle (McCaughy et al., 2010; Ryan et al., 2011). Among the dairy cattle breeds, prevalence was reported to be higher in Holstein than other breeds (McCaughy et al., 2010).

### **Season, environment and management factors**

Seasonal variation is observed in the occurrence of human Q fever. This variation, however, varies according to geographical region. But most cases of Q fever have been



reported in the spring or in early summer (Maurin and Raoult, 1999). Human Q fever has been shown to have a relationship with rainfall rather than season, based on a study in France (Gardon et al., 2001). A high prevalence of Q fever was observed among people living in close proximity to infected animals or in areas with a high livestock density (Maurin and Raoult, 1999; Smit et al., 2012).

Seasonality in the occurrence of *C. burnetii* infection was also reported in animals. In Japan, most of the Q fever cases in animals were reported in winter (Hellenbrand et al., 2001). On the other hand, in Germany most of the animal cases were reported in summer (Hellenbrand et al., 2001) and in autumn in Cyprus (Cantas et al., 2011). Increasing animal density increases the infection load in the environment, and is therefore, a potential risk factor of *C. burnetii* infection. Several studies in cattle identified that seroprevalence increases with an increasing herd size (McCaughey et al., 2010; Paul et al., 2012). Flock size is reported to have a similar effect in sheep (Kennerman et al., 2010). Several management factors such as housing systems, isolation of a newly introduced animal etc., may also contribute to the seroprevalence of *C. burnetii* infection in animals (Capuano et al., 2001; Paul et al., 2012).

## **Clinical manifestation in humans and animals**

*C. burnetii* infection can produce both acute and chronic forms of clinical manifestations in humans. However, in most cases (60%) infection remains asymptomatic and among the symptomatic cases, only a few patients develop a severe illness (Arricau-Bouvery and Rodolakis, 2005; Maurin and Raoult, 1999). The incubation period of Q fever is 2-3 weeks, depending on the route of infection (Maurin and Raoult, 1999). Clinical signs of acute Q fever are nonspecific and vary among patients. A self-limited febrile illness is probably the most frequent manifestation in most clinical cases, which is accompanied by severe headaches, myalgia, arthralgia and a cough (Tissot-Dupont and Raoult, 2007). A prolonged fever, which may reach 39-40°C, usually stays for 2-4 days and then gradually decreases to a normal level through the following 5-14 days (Maurin and Raoult, 1999). Atypical pneumonia is another common symptom of acute Q fever. Pneumonia is mild in most cases being characterised by a dry cough, fever, and minimal respiratory distress. Patients may also develop hepatitis with hepatomegaly, but without jaundice, subclinical hepatitis and granulomatous hepatitis with a prolonged fever (Maurin and Raoult, 1999). Generally, hepatitis develops in young immunosuppressed patients, whilst pneumonia is often seen in older patients (Fournier et al., 1998). Myocarditis is found in 2% of patients with the acute illness, which may be accompanied by pericarditis (Fournier et al., 2001). Skin rashes and neurologic disorders such as meningoencephalitis or encephalitis, lymphocytic meningitis and peripheral neuropathy have also been observed in acute Q fever cases (Bernit et al., 2002; Maurin and Raoult, 1999). Spontaneous abortion, intrauterine foetal death, premature delivery or retarded intrauterine growth may occur in women that become infected during pregnancy (Carcopino et al., 2007). However, recent studies in Denmark did not find any evidence of association between *C. burnetii* antibody positivity and spontaneous abortion, preterm birth or other adverse pregnancy outcomes (Nielsen et al., 2013). Pregnant woman may become chronically infected and abort in subsequent pregnancies (Maurin and Raoult, 1999).

Mortality is a rare outcome of the acute form of the disease. However, severe respiratory distress and myocarditis may lead to death (Fournier et al., 2001; Tissot-Dupont et al., 1992).

An infection which lasts for more than six months after the onset is defined as chronic Q fever. This happens in less than 5% of cases (Raoult and Marrie, 1995). The major clinical manifestation of this form of the disease is endocarditis (Tissot-Dupont and Raoult, 2007). It occurs in 60-70% of all chronic cases (Maurin and Raoult, 1999). The case fatality of Q fever endocarditis is less than 10% when patients are treated with antibiotics (Maurin and Raoult, 1999). The aortic and mitral valves are usually affected (Maurin and Raoult, 1999). Unspecific signs like intermittent fever, cardiac failure, weakness, fatigue, weight loss or anorexia may be present. Other manifestations are osteomyelitis, osteoarthritis, chronic hepatitis, hepatomegaly, splenomegaly, digital clubbing, purpuric rash and an arterial embolism (Maurin and Raoult, 1999; Tissot-Dupont and Raoult, 2007; Williams and Sanchez, 1994).

The term coxiellosis is considered to be more appropriate than animal Q fever, as most cases of animal infection are asymptomatic (Lang, 1988). The organism is found in the blood, lungs, liver and spleen during acute experimental infection, whereas chronically infected animals persistently shed bacteria in their faeces and urine. Infection in most domestic animals remains unrecognised. Coxiellosis is considered a cause of abortion and reproductive disorders in domestic animals (Williams and Sanchez, 1994). There is scientific evidence to support the hypothesis that *C. burnetii* can induce epidemics of reproductive failure in sheep and goats, but not in cattle (Agerholm, 2013). Reproductive disorders in domestic animals include endometritis, metritis, stillbirth, reduced birth weight and infertility. In a number of studies, *C. burnetii* bacteria or antibodies were identified from clinical cases of stillbirth, retained placenta, infertility, endometritis and metritis; however, the evidence was lacking for their association with *C. burnetii* infection in cattle (Bildfell et al., 2000; Cabassi et al., 2006; Garcia-Ispuerto et al., 2012; Muskens et al., 2011; Muskens et al., 2012). The herd level perinatal mortality and rate of still birth were not associated with the level of *C. burnetii* antibodies in bulk tank milk in Danish dairy cattle (Nielsen et al., 2011). If abortions occurred, then the rates generally remained low; however, it ranged from 3-80% (Marrie, 2007). Abortion rate is comparatively higher in ewes and goats than in cows. Abortion is usually observed in late pregnancy in both ewes and cattle (Lang, 1990). In most abortive cases, the aborted foetus appears normal. Discoloured exudate and intracotyledonary fibrous thickening may be observed in an infected placenta. Severe myometrial inflammation and metritis are the frequently observed clinical manifestations in goats and cows, respectively (Arricau-Bouvery and Rodolakis, 2005).

## Diagnosis

There are no specific clinical signs of *C. burnetii* infection in humans and animals. Therefore, laboratory diagnosis is the only way to confirm the disease. Since *C. burnetii* is highly infectious, biosafety level 3 laboratories and experienced laboratory personnel are required to handle the contaminated specimens (Fournier et al., 1998). Several human specimens have to be taken in order to detect *C. burnetii*. However, the clinical condition

of the patient determines the type of specimen. Aborted placenta and amniotic fluid are the best specimens for detection and identification of *C. burnetii* in animals. Milk, faeces, urine and uterine discharge can also be used to identify shedder animals. Nowadays, milk and blood samples are widely used to identify seropositive animals in epidemiological studies.

### **Culture and animal inoculation**

Standard biological media are not suitable for the growth of *C. burnetii* (Maurin and Raoult, 1999). Therefore, isolation of bacteria is done using the shell-vial cell culture techniques or culture in the yolk sacs of embryonated eggs. Guinea pigs, although rarely used nowadays, develop a fever 5-8 days after intraperitoneal inoculation (Woldehiwet, 2004). Further isolation can be done by culturing bacteria in the cell lines or yolk sac from the heavily infected spleen of the inoculated guinea pig.

### **Staining and immunodetection**

Fixed impression or smears prepared from aborted placenta, uterine discharge and other secretions from cows, ewes and goats can be stained with Machiavello or modified Ziehl Neelsen stains to detect the organisms (Angelakis and Raoult, 2010; Woldehiwet, 2004). Antigen detection from impression smears or histological sections can be done by direct or indirect immunofluorescence or immunohistochemical techniques (Woldehiwet and Aitken, 1993), or by fluorescence in situ hybridization (Hansen et al., 2011).

### **Serology**

#### ***Complement fixation test (CFT)***

A Complement fixation test (CFT) was a widely used laboratory technique for diagnosis of human Q fever in the past, and it is still an OIE recommended test for *C. burnetii* infection in animals. The advantage of CFT is that it does not require host species-specific antibodies (Peter et al., 1985). This test is highly specific, but weakly sensitive (Kovacova et al., 1998; Peter et al., 1985). Moreover, CFT cannot detect early stages of infection as the complement fixing antibodies do not appear in exposed individuals in early stages of the infection (Peter et al., 1985). Therefore, samples from both convalescent and acute phases are required to accurately diagnose the infection. It has been shown that the antigens used in CFT often fail to identify seropositive sheep and goats (Kovacova et al., 1998).

#### ***Indirect immunofluorescence assay (IFA)***

Indirect immunofluorescence assay (IFA) has been widely used and remains as a frequently used method for diagnosis of human infection (Bosnjak et al., 2010, Maurin and Raoult, 1999; Nielsen et al., 2013, Tissot-Dupont et al., 1994). It requires a very small amount of antigens and can detect IgG, IgM and IgA against phase I and phase II antigens; and therefore, acute and chronic infections can be differentiated by an IFA test (Tissot-

Dupont et al., 1994). In an acute case, antibodies can be detected 7-15 days after the onset of clinical signs. An elevated level of IgG against phase II antigens ( $\geq 200$ ) and a much lower level of IgM against phase I antigens ( $\geq 50$ ) indicate an acute infection (Tissot-Dupont et al., 1994). A high level of IgG against both phases I and II and a slightly elevated level of IgA against phase I antigens, although not exclusively predictive, can be detected in a chronic infection. An IgG titre  $\geq 800$  and an IgA titre  $\geq 50$  against phase I antigens are often found in chronic infections (Tissot-Dupont et al., 1994). IFA is a species specific test and is not often used for diagnosis of *C. burnetii* infection in animals.

### **Enzyme-linked immunosorbent assay (ELISA)**

Several studies in humans have shown that enzyme-linked immunosorbent assay (ELISA) has a higher sensitivity than CFT and IFA (Peter et al., 1985; Peter et al., 1987; Soliman et al., 1992), and was recommended as a useful diagnostic tool for seroepidemiological studies (Peter et al., 1987). ELISA can detect antibodies against both phase I and phase II antibodies (Maurin and Raoult, 1999). This test has a higher sensitivity than the CFT in animal studies (Emery et al., 2012; Horigan et al., 2011; Rousset et al., 2010), and is a quick diagnostic technique (Rousset et al., 2010). It allows the testing of a large number of samples at the same time and is a popular tool for seroepidemiological studies in animals (Rousset et al., 2010).

### **Polymerase chain reaction (PCR)**

Recently, several polymerase chain reaction (PCR) techniques have been developed and successfully used to detect *C. burnetii* DNA in cell cultures and in clinical samples (Berri et al., 2000; Brennan and Samuel, 2003; Fenollar et al., 2004; Fenollar and Raoult, 2007). This technique is highly sensitive and specific, and is a rapid tool for *C. burnetii* detection (Rousset et al., 2010). PCR also has improved the diagnostics of Q fever in veterinary science (Berri et al., 2003). Insertion sequence IS1111 is one of the most commonly used primers in real time PCR for detection and quantification of bacterial DNA (Angen et al., 2011; Berri et al., 2000). Primers specific for the superoxide dismutase (*sodB*) gene; *com1* encoding a 27 kDa outer membrane protein; heat shock operon encoding two heat shock proteins (*htpA* and *htpB*); isocitrate dehydrogenase (*icd*); and macrophage infectivity potentiator protein (*cbmip*) are also used in PCR. A high degree of specificity has been shown by the primers specific to some plasmid mediated genes (*QpRs*, *QpH1*, *cbbE*) (Willems et al., 1994), primers specific to the *htpAB*-associated repetitive element (Willems et al., 1994), superoxide dismutase gene (Stein and Raoult, 1992) and the 16s rRNA (Willems et al., 1994).

### **Treatment**

Doxycycline (200 mg daily for 14 day) is the recommended drug for acute cases of Q fever in adults and children (Maurin and Raoult, 1999). Alternatively, fluoroquinolones can also be used for the patients with meningoencephalitis. Cotrimoxazole and rifampin are the drugs of choice for the patients allergic or contradicted to tetracyclines (Maurin and Raoult, 1999). Long-term (>5 weeks) use of cotrimoxazole with folinic acid is recommended for pregnant women (Carcopino et al., 2007). It has been shown that this

treatment is protective against placental infection, obstetric complications and foetal death. Doxycycline (100 mg/day) and hydroxychloroquine (600 mg) are the recommended treatment for chronically infected patients (Carcopino et al., 2007). This treatment, however, must be continued for more than 18 months.

Two injections of oxytetracycline (20 mg per kg body weight) in the last trimester of pregnancy are usually recommended for animals, although this may not completely suppress abortions or stop bacterial shedding during parturitions (Berri et al., 2007).

## Options for prevention and control

Like other zoonoses, control of Q fever in humans is largely dependent upon the control of *C. burnetii* infection in animals. The control of infection in domestic animals requires knowledge about the factors contributing to the introduction and spread of the infection. However, only a few risk factor studies have been conducted. Based on the available knowledge and experience from the recent outbreaks in the Netherlands, it can be concluded that legislative measures (e.g. culling of animals, banding movement and transport, mandatory vaccination) (Roset et al. 2011b) and improved biosecurity measures (e.g., avoiding contact between farm animals and visitors, quarantine of newly introduced animals and improved hygienic precautions of farm personnel) may also play roles in controlling *C. burnetii* infection (Paul et al., 2012). Other control measures which can be implemented to reduce the number of infected animals and environmental contamination are: lime or calcium cyanide 0.4% treatment of manure (Angelakis and Raoult, 2010); safe disposal of aborted fetuses, foetal membranes and contaminated bedding materials to avoid their ingestion by domestic or wild carnivores (Woldehiwet, 2004); thorough cleaning and disinfection of utensils and transporting vehicles (Woldehiwet, 2004). Appropriate measures should be taken to reduce the tick burden in the environment. Appropriate biosecurity measures should be implemented in animal farms and industries, and also in diagnostic laboratories to prevent the personnel from acquiring infection (Woldehiwet, 2004). Avoiding exposure to raw milk and the pasteurisation of milk and milk products can contribute to the reduction of Q fever burden in humans.

In the recent outbreaks in some EU Member States, a wide range of control measures were pursued in order to minimise the exposure to humans and to reduce spill-over from animals to humans. These included the restriction of infected farm visits in the Netherlands (EFSA, 2010; Roset et al. 2011b; Van den Brom and Vellema, 2009), avoiding human gatherings in high-risk areas (EFSA, 2010; Panaiotov et al., 2009); the closing of schools during an outbreak during 2004 in Bulgaria (Panaiotov et al., 2009); banning blood donation programs in affected areas in France during 2002 and 2007, and in Germany during 2005 (EFSA, 2010; Georgiev et al., 2013; INVS, 2009); shifting infected herds/flocks from human locations in Bulgaria during 2004 (Panaiotov et al., 2009); and introduction of a ban on animal movements (Georgiev et al., 2013). Furthermore, changes in the farming practices including manure management such as covering and natural composting or ploughing of manure, treating manure with lime (in the Netherlands) or calcium cyanide (in France and Germany) (EFSA, 2010; Roset et al. 2011b; Van den Brom and Vellema, 2009, Vellema et al. 2010) and the removal of animal birth and abortion products (EFSA, 2010; Georgiev et al., 2013), disinfection of infected premises including

paths and general environments of holding in Bulgaria during 2004 (Panaïotov et al., 2009); and the implementation of a farm animal breeding ban in the Netherlands during 2007–2010 (EFSA, 2010; Roset et al. 2011b), have also been practised. Moreover, in 2010, more than 50,000 pregnant small ruminants from PCR bulk tank milk positive farms were culled in the Netherlands with an aim of reducing the shedding of *C. burnetii* in order to reduce the human exposure from environmental contamination. This culling program was supplemented by a program of repopulation with immunised animals from PCR bulk tank milk negative farms only. A compensation scheme was also available for the farmers affected by the culling program (Roest et al., 2011a; Roest et al., 2011b). However, the effectiveness of different control measures remains uncertain. It has been reported that the prevalence of *C. burnetii* in an infected herd usually declines over time, even without taking any control measures. This is probably due to the ‘natural’ immunisation of susceptible animals (Georgiev et al., 2013). However, enough strong scientific evidence is still unavailable to make a final conclusion.

Q fever is considered to be a professional hazard, therefore, vaccination is primarily considered for those who are professionally at risk such as livestock farmers, producers of animal products, veterinarians and laboratory workers (Maurin and Raoult, 1999). Three types of vaccines can be used for preventing Q fever in humans: the attenuated live vaccine, a chloroform-methanol residue extracted vaccine and other extracted vaccines and the whole-cell formalin-inactivated vaccine. A vaccination program for people at risk from chronic Q fever, such as patients with cardiac valve disease, aortic aneurisms and vascular prostheses was launched in the Netherlands in July 2010, which commenced in January 2011, after the Q fever outbreak in the Netherlands had subsided (Georgiev et al., 2013; van der Hoek, 2012). Vaccinations have been shown to reduce abortion, shedding of *C. burnetii* and the occurrence of infection in animals (Arricau-Bouvery and Rodolakis, 2005; Hogerwerf et al., 2011; Kovacova and Kazar, 2002). It has been recommended that vaccination has to be sustained for at least several years (Astobiza et al., 2011; de Cremoux et al., 2012). Outbreak vaccination, i.e. vaccinating herds that are already infected (Astobiza et al., 2011; Guatteo et al., 2008) or otherwise under high infection pressure (de Cremoux et al., 2012), is less effective than regular vaccinations. Phase I vaccine is recommended for animals, as it is more protective than the phase II vaccine. The vaccination of animals was implemented during the outbreak in France during 2009; and in the Netherlands during 2007–2010 (Hermans et al., 2011; Rodolakis, 2009; Roest et al., 2011b). In the Netherlands, the vaccination program was initiated in October 2008, through a voluntary scheme involving dairy sheep and dairy goats on farms with more than 50 goats or sheep, petting zoos and nursing farms in a restricted high risk zone. This program was made mandatory in January 2010, subsequently introduced in an enlarged area (EFSA, 2010; Georgiev et al., 2013; Roest et al., 2011b). Nationwide mandatory vaccination coverage was achieved in 2011, which also included small ruminants attending animal shows (EFSA, 2010).

## Conclusion

*C. burnetii* is a widely distributed bacterium with zoonotic potentials. Since its first discovery in 1937, knowledge about this bacterium and the disease Q fever has increased quite a lot. However, there are still knowledge gaps which require further scientific studies. *C. burnetii* infection in domestic animals has been reported from almost all countries in

the world including Denmark. Despite this, very little is known about the pathogenesis of *C. burnetii* infection in domestic animals. It is also known to be a cause of reproductive failure in domestic animals, including cattle. However, available literature has failed to prove the association between infection and the reproductive failures in cattle, and this area demands more systematic studies. Although there is a long history of existence, the complete epidemiology of *C. burnetii* infection in cattle has not been explored sufficiently. In light of exploring the epidemiology, the following chapters of this thesis will discuss the frequencies and risk factors of *C. burnetii* antibody positivity in Danish cattle and the potential of diagnostic tests used to identify the positive animals.

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## **CHAPTER 3**

### **HERD PREVALENCE OF *COXIELLA BURNETII* SEROPOSITIVITY IN DANISH DAIRY CATTLE**



## **CHAPTER 3**

# **INCREASING PREVALENCE OF *COXIELLA BURNETII* SEROPOSITIVE DANISH DAIRY CATTLE HERDS**

Jens F Agger and **Suman Paul**

Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

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## Abstract

A study based on bulk tank milk samples from 120 randomly selected dairy cattle herds was conducted to estimate the prevalence of *Coxiella burnetii* seropositive dairy herds to describe the geographical distribution, and to identify the risk factors. Using the CHEKIT Q-fever Antibody ELISA Test Kit (IDEXX), the study revealed a prevalence of 79.2% seropositive herds, 18.3% seronegative herds and 2.5% serointermediate herds, based on the instructions provided by the manufacturer. Multifactorial logistic regression showed statistically significant associations ( $P<0.01$ ) between *C. burnetii* seropositivity and increasing herd size (OR=1.02 per cow increment) and an increasing regional average number of cattle per dairy herd (OR=1.02 per animal increment). The regional average number of cattle herds per square kilometre was borderline significantly related to the occurrence of seropositive dairy herds ( $P=0.06$ ). The results show an increased prevalence of seropositive dairy herds since the latest survey in 2008 and an adverse impact of increasing herd size and cattle density on the risk of seropositivity.

**Key words:** *Coxiella burnetii*; seroprevalence; herd size; cattle and herd density; bulk tank milk.

## Introduction

Repeated surveys of the frequency of infectious diseases are necessary for farmers, agricultural organizations and veterinary services to evaluate the needs for implementing disease control procedures. *Coxiella burnetii*, an obligate intracellular bacterium and a zoonotic agent that may cause Q fever in animals and humans, occurs in cattle almost worldwide (Angelakis and Raoult, 2010; Maurin and Raoult, 1999). The prevalence of *C. burnetii* antibody positive Danish dairy herds in 2008 was 59% (Agger et al., 2010) based on bulk tank milk samples (BTM) from 100 randomly selected herds. Since then publications indicate increasing prevalence in several European countries. Thus, *C. burnetii* infection had been detected in 13 member states of the European Union in 2010 (European Food Safety Authority (EFSA), 2012). Publications based on BTM samples representative of the target populations of dairy herds reported the prevalence of antibody positive dairy herds to be 79% in the Netherlands (Muskens et al., 2011), 38 % in the Republic of Ireland (Ryan et al., 2011), 65% in Northern Ireland (McCaughy et al., 2010), 67% in Northern Spain (Astobiza et al., 2012), and 71% in Wallonia, Belgium (Czaplicki et al., 2009). Our objectives were therefore, in a repeated study, to estimate the prevalence of *C. burnetii* seropositive dairy herds, to describe the geographical distribution, and identify risk factors using the herd as the analytical unit based on BTM samples.

## Materials and methods

In a cross sectional designed survey we randomly selected 120 dairy herds to be tested for the presence of *C. burnetii* antibodies in BTM samples. The sample size was calculated using the formula  $n = Z^2 pq / l^2$  with an assumed prevalence  $p = 0.50$  and an allowable error on the estimate of  $l = 0.10$  at the 95% confidence level. Although we had a prior knowledge of  $p = 0.59$  (Agger et al., 2010) we used  $p = 0.50$  to maximize the sample size. The calculated sample size was 97 herds. Taking the possibility of losing samples during collection and laboratory handling into account, we decided to include 120 herds. However, no samples were lost. The inclusion criteria were that the herd was delivering milk to a dairy plant at the time of selection in July 2012 and that the herd participated in a milk recording scheme and had all lactating cows milk yield controlled at least 11 times per year. All Danish herds which met the inclusion criteria were assigned a random number between 0 and 1 (SAS function Ranuni (0)), and the 120 herds with the lowest numbers were included in this study. The samples were tested at the Eurofins Steins Laboratorium A/S Denmark for antibodies against *C. burnetii*, using the commercially available CHEKIT Q-fever Antibody ELISA test (IDEXX, Liebefeld-Bern, Switzerland) based on *C. burnetii* inactivated phase 1 and 2 antigens following the manufacturer's instructions. The optical density (OD) of each sample was corrected by subtracting the OD of the negative control. The results were expressed as sample-to-positive values and estimated as  $S/P = [(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}}) \times 100]$ . According to the manufacturer,  $S/P \geq 40\%$ ,  $S/P < 30\%$  and results in the interval  $30\% \leq S/P < 40\%$  were considered as positive, negative and intermediate, respectively. However, for the purpose of risk factor analysis in logistic regression, we dichotomised the test results as positive for samples with  $S/P \geq 40\%$ , and as negative for samples with  $S/P < 40\%$ . Supplementary herd information for the year 2012 was extracted from the Danish Cattle Database.

The data was analysed in SAS. The prevalence of positive, negative and intermediate results with the confidence interval was estimated using the Proc SURVEYFREQ command. The Chi-square ( $\chi^2$ ) test was used to compare the prevalence found in this study with the prevalence in 2008. An association between herd antibody status and herd size, dominant milk breed type, animal purchase, bulk tank milk somatic cell count, average fat and protein percentage, average milk delivery to dairy plant per cow, herd type (organic/conventional), regional number of cattle herds per km<sup>2</sup> (all cattle types) and regional average number of cattle per dairy herd, and regional average number of cattle per cattle herd (all cattle types), were tested by univariable logistic regression followed by multivariable logistic regression with backward elimination of non-significant variables. Statistical significance of the covariates was assessed using the likelihood ratio test based on  $P \leq 0.05$ . Collinearity among the selected variables was assessed, and variables with correlation coefficients  $|\rho| \leq 0.5$  were considered for inclusion in the final model. The values of Hosmer-Lemeshow goodness of fit test were used to validate the final model.

Twenty nine of the 120 BTM samples were tested twice using two separate ELISA plates to validate the precision of the diagnostic test. This was evaluated in a Pearson correlation analysis considering S/P values as measured on a continuous scale and as a categorised variable (S/P $\geq$ 40 as positive and S/P<40 as negative), estimating Kappa ( $\kappa$ ) for the agreement between the test results and McNemar's test.

## Results and discussion

Descriptive analysis showed that the S/P values of the BTM samples ranged from 1 to 293 (Figure 1). The apparent prevalence of positive, negative and intermediate herds was 79.2%, 18.3% and 2.5%, respectively. Table 1 represents the summary statistics for the three test categories. The prevalence of positive herds in the present study was significantly higher ( $P<0.01$ ) than the estimated prevalence (59%) in the study in 2008 (Agger et al., 2010).

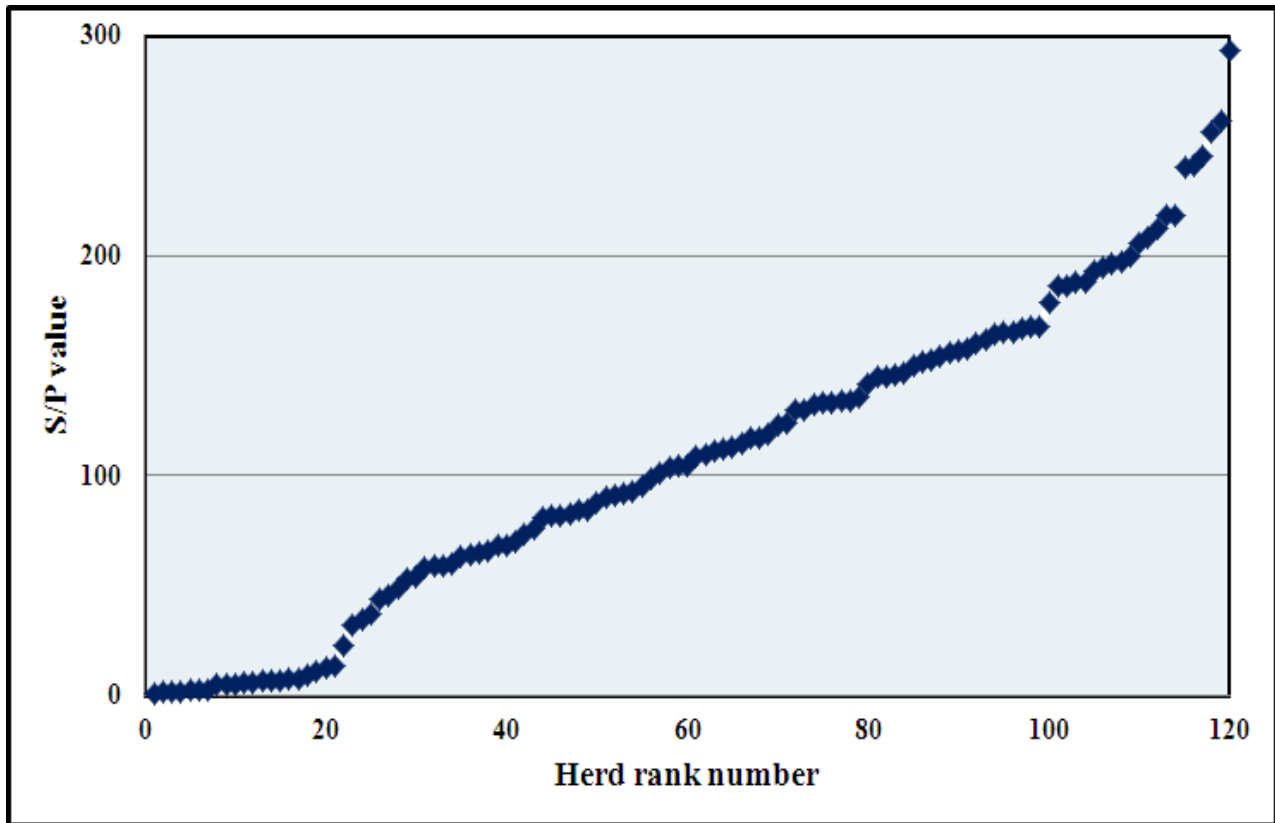
**Table 1:** Summary statistics of *Coxiella burnetii* antibody status of 120 randomly selected dairy herds in July 2012.

Herd category	No. of herds	Apparent prevalence (95% CI)	Mean S/P value $\pm$ SE	Range of S/P value
Positive	95	79.17 (71.80; 86.54)	132.80 $\pm$ 5.79	44.00 – 293.00
Negative	22	18.33 (11.31; 25.36)	6.82 $\pm$ 1.08	1.00 – 23.00
Intermediate	3	2.50 (0.00; 5.33)	34.67 $\pm$ 1.45	32.00 – 37.00

Herd size, average milk delivery per cow to dairy plant and regional average number of animals per dairy herd and regional average number of cattle per cattle herd (all cattle types) were all found to be significant in univariable analyses ( $P<0.05$ ). A regional number of cattle herds per km<sup>2</sup> (all cattle types) was borderline significant ( $P=0.06$ ). In the final multivariable model (Table 2), increasing herd size and increasing regional average number of cattle per dairy herd were significantly associated with *C. burnetii*



seropositivity. The Hosmer-Lemeshow value confirmed good fit to the data of the final model ( $P=0.74$ ). The regional number of cattle herds per km<sup>2</sup> (all cattle types) and the distribution of the sampled herds are presented in Figure 2.



**Figure 1:** Array of antibody S/P values to *Coxiella burnetii* in bulk tank milk samples from 120 randomly selected Danish Dairy herds in July 2012.

**Table 2:** Multivariable logistic regression model of risk factors associated with *Coxiella burnetii* antibody status (positive or negative).

Variables	Odds ratio (95% confidence interval)	P-value
Herd size <sup>a</sup>	1.02 (1.01-1.03)	<0.001
Regional average number of cattle per dairy herd <sup>a</sup>	1.02 (1.00-1.03)	0.02

<sup>a</sup> Odds ratio calculated per unit change in measurement.

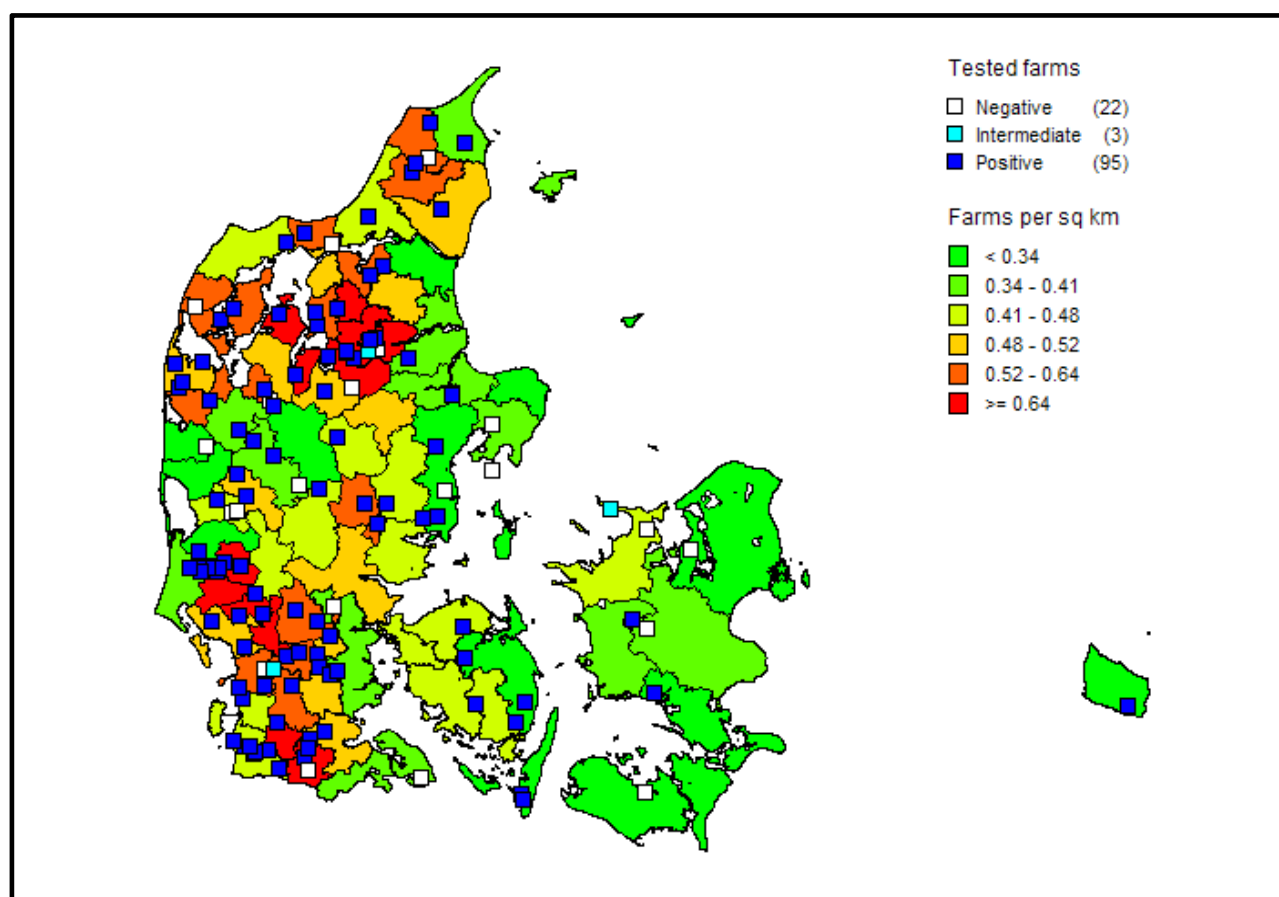
A comparison of duplicate test results of 29 BTM samples in two separate ELISA plates showed a high correlation between the two test results ( $r^2=0.96$ ). When categorising the 29 duplicate tests as positive or negative, there was full agreement between the test results ( $\kappa =1.00$  and  $P=1$ ). This shows a high precision of the laboratory procedure.

The prevalence of 79% *C. burnetii* seropositive Danish dairy herds in 2012 is a clear increase compared to the prevalence of 59% in 2008. This corresponds well to an

increasing trend in results from other European countries, as reviewed above and in reviewed literature in Agger et al. (2010).

The cattle herd density is highest in the south western and in the north western areas of Denmark. These are also the areas where most of the sampled herds are located. However, there is no clear clustering of herd status compared to density. Garcia-Seco et al. (2011) did not find clustering of positive herds in a study in the Madrid region in Spain. However, Beaudeau et al. (2012) in a study of BTM samples from 2600 dairy herds in the region of western France identified some clustering, indicating a wind borne impact on the spread of the infection.

Like in our study, Ryan et al. (2011) and McCaughey et al. (2010) also found a positive relationship between increasing herd size and test positivity in BTM samples. A recent Danish multilevel study with cows as the analytical unit also found an increasing risk of seropositive cows with increasing herd size (Paul et al., 2012).



**Figure 2:** Regional number of cattle herds per km<sup>2</sup> (all cattle types) and distribution of 120 randomly selected Danish dairy herds tested for antibodies against *Coxiella burnetii* in July 2012.

The study is based on a random sample of herds and thus, the results are considered valid for the current prevalence of *C. burnetii* seropositive dairy herds in Denmark.

However, the sample of 120 herds may be slightly too small for a more detailed cluster analysis, and that is why we have only used simple mapping of the study herds compared to the herd density (Figure 2).

It is concluded that the prevalence of seropositive dairy herds has increased since the latest survey in 2008, and that there is an adverse impact of increasing herd size and of the regional average dairy herd size on the risk of a dairy herd being seropositive.

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## **CHAPTER 4**

# **RISK FACTORS FOR *COXIELLA BURNETII* IN DANISH DAIRY CATTLE**



## CHAPTER 4 A

### RISK FACTORS FOR COXIELLA BURNETII ANTIBODIES IN BULK TANK MILK FROM DANISH DAIRY HERDS

Jens F Agger<sup>a</sup>, **Suman Paul**<sup>a</sup>, Anna-Bodil Christoffersen<sup>b</sup>, Jørgen S Agerholm<sup>a</sup>

<sup>a</sup> Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

<sup>b</sup> National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.

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## Abstract

The aim was to identify risk factors associated with *Coxiella burnetii* antibody positivity in bulk tank milk (BTM) samples from 100 randomly selected Danish dairy cattle herds. Antibody levels were measured by an enzyme-linked immuno-sorbent assay. Before testing the herds, the farm managers were interviewed about hired labour, biosecurity, housing and herd health during the 12 months prior to the study. Variables considered important for *C. burnetii* antibody positivity in multivariable logistic regression analysis included the sharing of machines between farms (OR=3.6), human contacts (OR=4.2), artificial insemination by other people than artificial insemination technicians (OR=7.7), herd health contract with the veterinarian (OR=4.3) and hygiene precautions taken by veterinarians (OR=5). In addition, herd size, hired labour, trading of cattle between farms, quarantine and use of calving and disease pens also showed significant association in univariable analysis. This study demonstrates that strict biosecurity is important for the prevention of infections with *C. burnetii*.

**Key words:** *Coxiella burnetii*; cattle; risk factors; biosecurity.

## Introduction

Control of infectious diseases in livestock is to a great extent based on prevention of introducing infectious microorganisms into susceptible populations. Therefore, identification of risk factors plays a key role in the management of biosecurity at farm level and there are increasing demands on development of biosecurity plans in livestock production in the European Union. Herd risk factors for Q fever, a zoonotic infection caused by the bacterium *Coxiella burnetii*, have not been well studied. Although *C. burnetii* has been found almost worldwide and despite rather high prevalences in many cattle populations (Guatteo et al. 2011), there is still need for knowledge on how to protect uninfected cattle herds from becoming infected. Thus the aim of this study was to identify risk factors for Danish dairy cattle herds having *C. burnetii* antibodies in bulk tank milk (BTM).

## Materials and methods

A cross sectional design was used to study 100 randomly selected dairy herds among the 4785 milk producing Danish dairy herds mandatorily listed in the Danish Cattle Database (Agger et al. 2010). Farmers of the selected herds were interviewed by telephone during 20-30 minutes using a standardized questionnaire with closed and semi-open-ended questions [ANNEX]. The questions concerned the use of hired labour, the housing system, general health of the herd, and farm management routines generally known to be of importance for herd biosecurity. A BTM sample from each herd was examined for *C. burnetii* antibodies using the commercial CHEKIT Q fever Antibody ELISA test kit (IDEXX, Liebefeld-Bern, Switzerland). The test was based on *C. burnetii* inactivated phase 1 and 2 antigens and the results were expressed as sample-to-positive values and estimated as  $S/P = [(OD \text{ sample} - OD \text{ negative control}) / (OD \text{ positive control} - OD \text{ negative control}) \times 100]$ . According to the manufacturer,  $S/P \geq 40\%$ ,  $S/P < 30\%$  and results in the interval  $30\% \leq S/P < 40\%$  were considered as positive, negative and intermediate respectively. However, in our risk factor analysis in logistic regression we dichotomized the test results as positive for samples with  $S/P \geq 40\%$  and as negative for samples with  $S/P < 40\%$ , as recommended by the manufacturer. The prevalence of seropositive herds was 59 %, as previously reported (Agger et al. 2010). Fisher's exact test was applied to test relationships between *C. burnetii* antibody status and all ordinal and dichotomized variables. To account for possible nonlinear relations, values of all continuous variables were categorized into biological meaningful classes when appropriate before further analysis. Variable associations with  $P \leq 0.25$  in univariable analyses were included in the following multivariable logistic regression. Backward elimination of nonsignificant variables ( $P > 0.05$ ) was used to select the final model, and the values of Hosmer-Lemeshow goodness of fit test were used to validate the models. Correlations among the exposure variables were checked by Spearman's correlation test to avoid collinearity. There was no significant ( $P \leq 0.05$ ) correlation and no variables were removed due to this.

## Results and discussions

Eighteen out of 49 variables that had  $P \leq 0.25$  in univariable analysis were included in the multivariable analysis. The final logistic regression model (Table 1) showed that the risk of

a seropositive BTM sample increased if the herd shared equipment (machines) with other farms, if cattle were in contact with visitors, if artificial insemination (AI) was done by other people than AI technicians, if the herd had a routine herd health contract with the veterinarian, and if hygiene precautions (changing boots and/or clothes, etc.) were not taken by the veterinarian before entering the herd. The Hosmer-Lemeshow value for goodness-of-fit of the final model was considered acceptable ( $P = 0.86$ ) (Hosmer and Lemeshow, 2000).

**Table 1:** Multivariable logistic regression model for risk factors jointly associated with bulk tank milk antibody positivity to *Coxiella burnetii* in 100 randomly selected Danish dairy cattle herds collected in February 2008.

Variables	Respondents		Odd Ratio (95% CI)	P-value*
	Total	No. (%) positive		
Sharing equipment (machines) with other herds				0.04
Yes	23	17 (73.91)	3.62 (1.03 - 12.76)	
No	77	42 (54.55)	1	
Animals' contact with visitors				0.01
Yes	70	47 (79.66)	4.17 (1.41 - 12.5)	
No	30	12 (20.34)	1	
Insemination by other than artificial insemination (AI) technician				0.01
Yes	13	12 (92.31)	7.69 (2.08 - 16.95)	
No	87	47 (54.02)	1	
Health contract with veterinarian				0.01
Yes	56	40 (71.43)	4.32 (1.51 - 12.36)	
No	44	19 (43.18)	1	
Hygienic precautions by veterinarian before entering the herd				0.004
No	61	40 (65.57)	5.00 (1.66 - 15.12)	
Yes	39	19 (48.72)	1	

\*P-value for the significant addition of the variable given the other variables in the model.

The final multivariable model (Table 1) primarily contains factors associated with introduction of infection into a herd. The factors: animal contact with human visitors from outside the farm, AI insemination by other people than AI technician, and herd health contract for routine health evaluation of the herd by the veterinarian were associated with increased antibody positivity (IAP). The most likely explanation is probably that such persons act as mechanical vectors carrying *C. burnetii* bacteria into the herd as stated in a review by Woldehiwet (2004). We found that AI done by other people than AI technicians increased the risk of BTM antibody positivity. Danish farmers who want to perform AI on their own cattle need authorization based on a course offered by the AI associations and

semen is provided by AI associations. So the finding is difficult to explain; also because consequent reduced access of AI technicians to the herd should probably have lowered the risk. Farms with a routine herd health contract with a veterinarian had higher OR of being antibody positive than farms without such a contract thus suggesting that the veterinarian might bring the bacterium into the farm. Hygiene precautions taken by veterinarian, i.e. changing boots and/or clothes were found significantly to reduce the risk of IAP. In a multilevel analysis of data from the same study, but with cow as the analytical unit, we also found that the hygienic precautions reduced the risk of antibody positivity (Paul et al., 2012). The similarity in results between cow and herd level analysis corresponds with our estimation of correlation between BTM antibody level and the within herd seroprevalence ( $R^2 = 0.36$ ;  $P < 0.001$ ) in a previous study (Angen et al., 2011) of a subsample of 12 of the same herds as in the present study. However, Taurel et al. (2012) only found a moderate correlation ( $R^2 = 0.15$ ) between BTM antibody level and within herd seroprevalence. In the present study we also observed that sharing farm equipment (machines) with other farms was significantly associated with IAP. Although our study indicates the importance of hygienic precautions in relation to personnel and equipment, Taurel et al. (2011) did not find such associations in French dairy cattle.

Herd size, stable type, number of workers, use of calving and disease pens, purchasing animals, and quarantine practice were also found to have positive associations with IAP in univariable analysis (results not shown). Other studies have also shown relationship between IAP and increasing herd size (McCaughey et al. 2010; Paul et al., 2012), and between IAP and loose housing system (Czaplicki et al., 2012; Paul et al., 2012) although a single study did not find relationship between IAP and housing system (Capuano et al., 2001). The hypothesis behind IAP and loose housing systems is that random movements of animals increase the probability of contact between infected and non-infected and increased contact with a contaminated environment and hence increased risk of transmission.

Selection bias in this study was minimized by random sampling and selection bias due to non-participation was considered negligible. Misclassification bias may result from unknown sensitivity and specificity of the ELISA used. However, the ELISA test for *C. burnetii* antibodies based on individual animal samples shows high sensitivity ( $Se = 0.86$ ) and specificity ( $Sp = 0.99$ ) (Paul et al., 2013).

In this study the following risk factors were found to be associated with increased risk of BTM antibody positivity: Herd size > 100 cows, increased number of people managing the animals; housing systems with cubicle house and deep bed house compared to tie stall house, use of calving and disease pens, purchase of animals, lack of quarantine of purchased animals, contact with farm personnel and visitors, herd health contract with the veterinarian, lack of basic hygienic precautions taken by veterinarian and sharing of machines. Proper management by farmers may help prevent the introduction of *C. burnetii* into a herd.

## List of abbreviations

AI: Artificial insemination; BTM: Bulk tank milk; ELISA: Enzyme-linked immunosorbent assay; IAP: Increased antibody positivity.

## **Competing interests**

JSA is editor-in-chief of Acta Veterinaria Scandinavia, but has not in any way been involved in or interacted with the review process or editorial decision making. The authors declare they have no competing interests.

## **Authors' contribution**

JFA, ABC and JSA designed the study and developed the questionnaire. JFA selected the herds and conducted the interviews. ABC was responsible for sampling of milk and conducted the laboratory analysis. SP and JFA conducted the analysis and drafted the manuscript. All authors commented and approved the final manuscript.

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## CHAPTER 4 B

### **Factors associated with *Coxiella burnetii* antibody positivity in Danish dairy cows**

**Suman Paul**<sup>a</sup>, Jens F. Agger<sup>a</sup>, Bo Markussen<sup>b</sup>, Anna-Bodil Christoffersen<sup>c</sup>, Jørgen S. Agerholm<sup>a</sup>

<sup>a</sup> Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

<sup>b</sup> Department of Basic Sciences and Environment, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, DK-1870 Frederiksberg C, Denmark.

<sup>c</sup> National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1790 Copenhagen, Denmark.





## Abstract

The aim of the study was to identify associations between the level of *Coxiella burnetii* (*C. burnetii*) antibodies in individual milk samples and cow and herd level factors in Danish dairy cows. The study, designed as a prospective cross sectional study with follow up, included 24 herds identified by a stratified random sampling procedure according to the level of *C. burnetii* antibodies in one bulk tank milk (BTM) sample at the beginning of the study. Ten herds were BTM positive, ten herds were BTM negative and four herds had an intermediate level. The samples were tested with an ELISA and results determined as S/P (sample to positive control) values. Three cross sectional studies of all lactating cows within each herd were then conducted during an 11 month follow up period with collection of a total of 5829 milk samples from 3116 cows. Each sample was tested with the same ELISA as used for BTM testing, and cows were considered test positive for S/P values  $\geq 40$ , and otherwise negative. Individual cow information was extracted from the Danish Cattle Database and herd information was obtained from a telephone interview with each farmer. From multivariable logistic regression analysis accounting for hierarchical structures in the data it was concluded that odds for seropositivity increased with Danish Holstein breed, increasing number of parity and high milk protein contents, but decreased with increasing milk yield and high milk fat contents. Cows were at a higher risk during summer than other seasons. Among the herd level factors, herd size, tie stall housing system, quarantine of newly purchased animals and good hygienic precautions taken by the veterinarian before entering into the stable were also significantly associated with reduced odds of *C. burnetii* antibody positivity. The prevalence of test positive cows was almost constant during the study period in herds which were initially BTM positive and BTM intermediate, whilst the prevalence of positive cows in a few of the initial BTM negative herds changed from almost zero to higher than 60%. This indicates that herd infections last quite long and that test negative herds may convert to positive due to a few latently infected cows or due to transmissions from other herds.

**Key words:** *Coxiella burnetii*; dairy cattle; ELISA; factors; epidemiology.

## Introduction

*Coxiella burnetii* (*C. burnetii*), the causative agent of Q fever, is an obligate, intracellular, pleomorphic bacterium (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005). It was first identified in 1937 in Australia (Derrick, 1937) and since then it has been reported as an endemic infection throughout the world except for New Zealand (Maurin and Raoult, 1999). Q fever is a zoonosis, and domestic ruminants such as cattle, sheep, and goats are considered the primary source of human infections (Maurin and Raoult, 1999; Angelakis and Raoult, 2010). Sixty percent of human cases are asymptomatic and if clinical disease develops it may progress from an acute to a chronic stage (Raoult et al., 2000). Infection in dairy cows mostly remains clinically unrecognized (Rodolakis, 2009). Infection in cattle has a long persistence (Lang, 1990) and infected cattle shed large amounts of bacteria via birth fluids and placenta during parturition (Rodolakis et al., 2007). Other common routes of bacterial shedding are milk (Willems et al., 1994), faeces (Guatteo et al., 2006), and urine (Heinzen et al., 1999). Infected cattle may shed bacteria via milk for a longer period (Rodolakis et al. 2007).

Several studies have shown that the risk of *C. burnetii* infection in ruminants varies with individual animal traits such as age and parity, breed, gender, level of milk production and lactation stage (McCaughey et al., 2010; Garcia-Ispuerto et al., 2011), and also with season (Yanase et al., 1997; Cantas et al., 2011). Herd related factors such as herd size, housing type and grazing practice can also act as contributing factors (Capuano et al., 2001; McCaughey et al., 2010; Ruiz-Fons et al., 2010; Ryan et al., 2011; Taurel et al., 2011).

In recent years a high prevalence of *C. burnetii* exposure has been reported in humans and domesticated animals in several European countries (Gilsdorf et al., 2008; McCaughey et al., 2010; Guatteo et al., 2011). Also in Denmark, an increasing number of Q fever cases has been reported in humans (Villumsen et al., 2009), and Agger et al. (2010) reported a high seroprevalence (59%) in BTM samples from 100 randomly selected Danish dairy cattle herds.

This study aimed at identifying associations between the level of *C. burnetii* antibodies in individual cow milk samples and cow-level and herd-level explanatory factors.

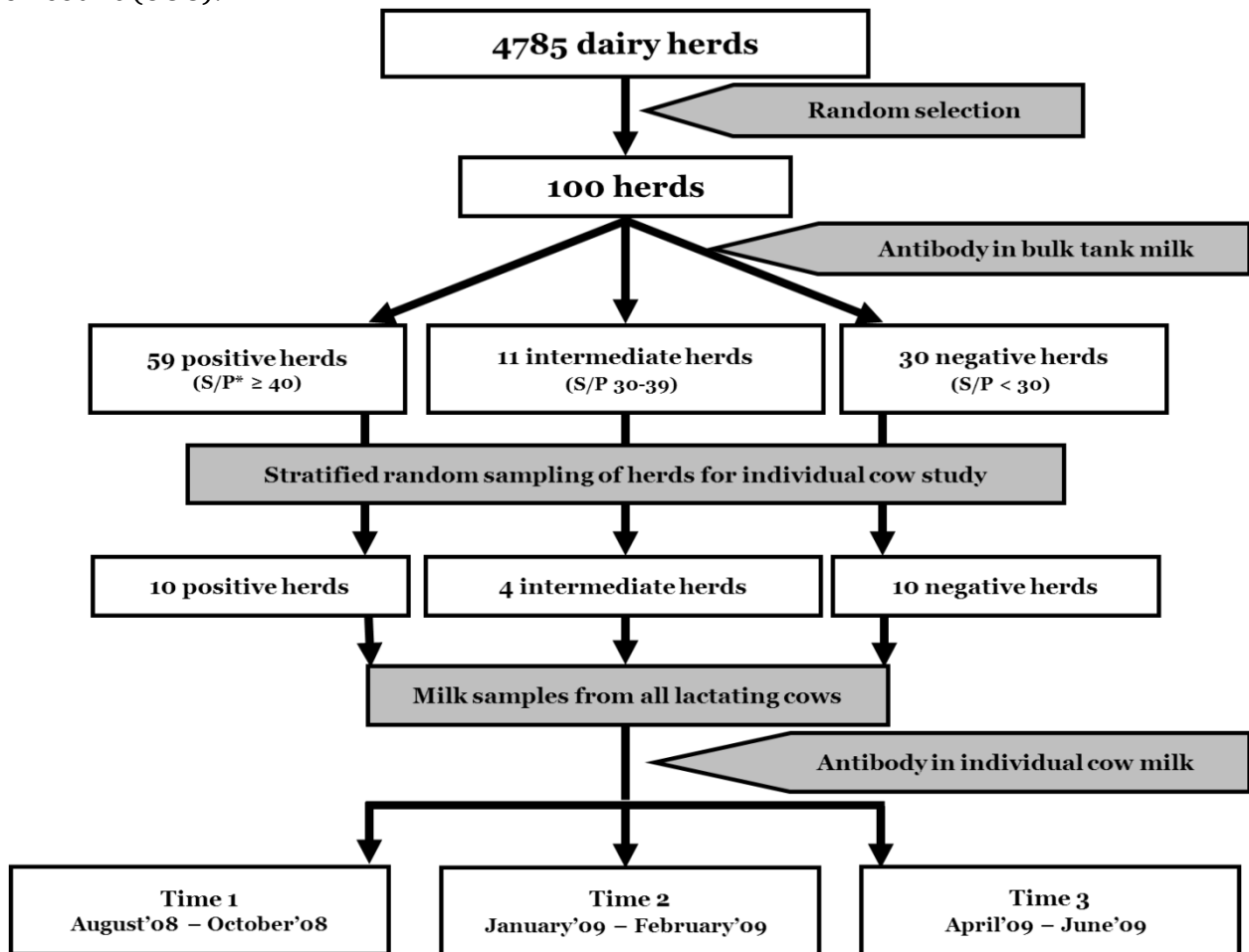
## Materials and methods

### Recruitment of study units and detection of antibodies

The study included 3116 lactating cows as study units from 24 dairy herds selected as explained in the following text. Initially 100 dairy herds were randomly selected from the Danish Cattle Database to estimate the prevalence of *C. burnetii* antibody positive herds based on one BTM sample (Agger et al., 2010). Milk samples were tested for the level of *C. burnetii* antibodies using the CHEKIT Q fever Antibody ELISA test kit (IDEXX, Liebefeld-Bern, Switzerland) based on *C. burnetii* inactivated phase 1 and phase 2 antigens. All samples were tested in duplicates and the optical densities (OD) of the samples were averaged. The results were expressed as S/P (sample to positive control) values calculated using the following equation:

$$S/P \text{ value} = \frac{OD_{\text{sample}} - OD_{\text{negative control}}}{OD_{\text{positive control}} - OD_{\text{negative control}}} \times 100 \quad (1)$$

The herds were classified according to the S/P level of antibodies into positive, intermediate and negative strata (see Figure 1). Following this initial study, ten BTM positive, ten BTM negative and four BTM intermediate herds from the three strata were randomly selected for a prospective cross sectional study with follow up. Thus, the sampling procedure was random, but the sample size was according to the economic conditions in the project. One goal was to contrast bulk tank milk positive and negative herds; however, the “intermediate” herds were included in the study due to suspected infection dynamics taking place. A total of 5829 individual cow milk samples from 3116 lactating cows in the 24 herds were collected at three time points during a period of 11 months from August 2008 to June 2009. All lactating cows in each herd were thus sampled one or two or three times at an interval of three to seven months. All samples were tested for the level of *C. burnetii* antibodies by the same ELISA test as used for BTM samples. The same milk samples were also tested for fat and protein contents and somatic cell count (SCC).



**Figure 1:** Diagram of design and data collection of a prospective cross sectional study with follow-up of associations between *Coxiella burnetii* antibody positivity and cow and herd level factors conducted from August 2008 to June 2009 involving 3116 cows from 24 Danish dairy herds. \* S/P = level of antibodies in the sample.

## Data collection and management

The study was based on data from interview with the farmers and on individual cow data from the Danish Cattle Database. The 100 farmers including the 24 farmers in the present study were telephone interviewed using a structured questionnaire. They were asked about labor, stable type, use of maternity and disease pens, animal's contact with other animals or humans, grazing practice, purchasing behaviour, quarantine of newly purchased animals, other biosecurity related factors like hygienic precautions taken by the herd veterinarian, the inseminator, the truck driver, visitors before entering the stable, use of foot bath and herd health during the 12 months prior to BTM sampling.

Individual cow information extracted from the Danish Cattle Database included records of reproductive, gastrointestinal, respiratory, udder and leg diseases treated by veterinarians. Some of the cow level variables were calculated using the information extracted from the cattle database. For example variable 'days in milk' was calculated by subtracting the date of last calving from the date of sample collection and the variable 'days in cow barn' was calculated by subtracting the date of first calving from the sampling date. Disease records were restricted to a three-month period around each sampling date i.e. from 45 days prior to the sampling date and until 45 days after the sampling date. If a cow, for example, was treated for any reproductive problem within 45 days before or after the sampling date, then the cow was considered to have a reproductive problem.

## Statistical analysis

At the cow level a milk sample with S/P value  $\geq 40$  was considered positive, and otherwise negative. Descriptive analyses of the qualitative and quantitative explanatory variables were performed to explore the distribution in relation to the dependent variable. When appropriate, continuous variables were categorized in order to account for possible nonlinear relations. Correlation among the explanatory variables was checked for multicollinearity and only one variable from each correlated group was used in the multivariable analysis. Univariable and multivariable logistic regression analyses were performed for quantifying the associations of explanatory variables with the dichotomous response variable using proc GLIMMIX in SAS 9.2. Hierarchical structure of the data was accounted for in the logistic regression analyses where 'herd' and 'cow' were used as random effects. Explanatory variables with p-values  $\leq 0.25$  in univariable logistic regression analyses were used in the multivariable model. Based on this criterion 11 cow level and 11 herd level variables were selected for further analyses. In multivariable analysis, a backward elimination procedure was used. All biologically meaningful interactions were also checked. The statistical significance of the explanatory variables was assessed by the likelihood ratio test and an explanatory variable with p-value  $\leq 0.05$  was considered to be statistically significant. The predictive ability of the final model was evaluated by the receiver operating characteristics (ROC) curve and area under the curve (AUC). Intra-class correlation coefficients (ICC) were determined to estimate the proportion of the variation explained by cows and herds. The stratum specific prevalences of *C. burnetii* seropositive cows were estimated for the three sampling time points. Differences in the cow level prevalence according to the time and stratum were assessed using a chi-square test.

## Results

### Estimation of associations

The results of the univariable logistic regression analysis with random effect of herd and cow are shown in Tables 1 and 2, respectively. The intrinsic factors breed, age in years, days in milk, milk yield, and number of days a cow had stayed in the cow barn were significantly associated with the level of *C. burnetii* antibodies in milk (Table 1). The odds of being positive were higher in Danish Holstein cows and in cows from “other breeds” (pooled breed group) compared to Jersey cows. ORs also increased with increasing days in milk. We found a negative association with milk yield and a positive association of seropositivity with udder problems (treatment for any problem in the udder for example mastitis and udder edema). There were no significant associations with other recorded diseases. Cows had higher odds in summer and in winter compared to autumn. Herd level factors, initial BTM antibody status, herd size, type of stable, use of maternity pen, contact with hoof trimmer, and hygienic precautions taken by the veterinarian before entering the stable were also significantly associated with the level of antibodies in milk in the univariable analyses (Table 2).

**Table 1:** Univariable logistic regression analysis of associations between *Coxiella burnetii* antibody positivity in cow milk samples and cow level factors adjusted for random effects of cow and herd in a prospective cross sectional study with follow-up conducted from August 2008 to June 2009 involving 3116 cows from 24 Danish dairy herds.

Variables	No. (%) of observations (n=5829)	No. (%) of (+)ve observations (n=1239)	Odds ratio (95% CI)**	P***
Breed				<0.01
Danish Holstein	3509 (60.10)	864 (69.73)	2.83 (1.98 - 4.04)	
Other	604 (10.36)	92 (7.43)	2.40 (1.57 - 3.66)	
Jersey	1716 (29.44)	283 (22.84)	1	
Age (year)*			1.10 (1.05 - 1.15)	<0.01
Parity				0.20
≥ 4	955 (16.41)	222 (17.93)	1.25 (1.01 - 1.56)	
3	918 (15.77)	203 (16.40)	1.14 (0.92 - 1.39)	
2	1581 (27.16)	325 (26.25)	1.04 (0.88 - 1.22)	
1	2367 (40.66)	488 (39.42)	1	
Days in milk †			1.03 (1.01 - 1.04)	<0.01
Milk yield (kg)*			0.99 (0.98 - 1.00)	0.04
Fat content (%)*			0.97 (0.92 - 1.02)	0.25
Protein content (%)*			1.07 (0.98 - 1.16)	0.14
Days in cow barn†			1.01 (1.00 - 1.01)	<0.01
Leg problems				0.07
Yes	147 (2.52)	49 (3.95)	1.35 (0.97 - 1.88)	
No	5682 (97.48)	1190 (96.05)	1	
Udder problems				0.05
Yes	638 (10.95)	164 (13.24)	1.20 (1.00 - 1.44)	
No	5191 (89.05)	1075 (86.76)	1	
Season				<0.01
Summer	2217 (38.03)	504 (40.68)	1.45 (1.25 - 1.69)	
Winter	2199 (37.73)	456 (36.80)	1.24 (1.06 - 1.44)	
Autumn	1413 (24.24)	279 (22.52)	1	

†Odds ratio calculated per 30 days change in measurement

\*Odds ratio calculated per unit change in measurement

\*\*CI, confidence interval

\*\*\*Variables significant at  $p \leq 0.25$  were included in the table

**Table 2:** Univariable logistic regression analysis of associations between *Coxiella burnetii* antibody positivity in cow milk samples and herd level factors adjusted for random effects of cow and herd in a prospective cross sectional study with follow-up conducted from August 2008 to June 2009 involving 3116 cows from 24 Danish dairy herds.

Variables	No. of herds	No. (%) of observations (n=5829)	No. (%) of (+)ve observations (n=1239)	Odds ratio (95% CI)**	P***
Initial bulk tank milk antibody status					0.02
Positive	10	3584 (61.49)	861 (69.49)	10.00 (1.82 - 55.02)	
Intermediate	4	707 (12.13)	167 (13.48)	10.90 (1.22 - 97.12)	
Negative	10	1538 (26.39)	211 (17.03)	1	
Herd size <sup>†</sup>				2.17 (1.19 - 3.99)	0.01
Type of stable					0.01
Loose housing	13	4328 (74.25)	1144 (92.33)	7.33 (1.69 - 31.88)	
Tie stall	11	1501 (25.75)	95 (7.67)	1	
Use of maternity pen					0.05
Yes	17	4929 (84.56)	1197 (96.61)	6.00 (1.03 - 35.03)	
No	7	900 (15.44)	42 (3.39)	1	
Sharing of machine					0.14
Yes	10	2209 (37.90)	430 (34.71)	3.28 (0.65 - 16.49)	
No	14	3620 (62.10)	809 (65.29)	1	
Purchase animal					0.20
Yes	18	4385 (75.23)	1035 (83.54)	3.36 (0.50 - 22.72)	
No	6	1444 (24.77)	204 (16.46)	1	
Visitors forbidden to enter the stable					0.10
No	22	5169 (88.38)	1226 (98.95)	7.43 (0.67 - 82.37)	
Yes	2	660 (11.32)	13 (1.05)	1	
Quarantine of newly purchased animals					0.10
No	15	3519 (60.37)	961 (77.56)	3.89 (0.77 - 19.73)	
Yes	9	2310 (39.63)	278 (22.44)	1	
Contact with professional hoof trimmer					0.03
Yes	15	3842 (65.91)	1041 (84.02)	5.38 (1.14 - 25.37)	
No	9	1987 (34.09)	198 (15.98)	1	
Contact with visitors					0.13
Yes	17	4466 (76.62)	1135 (91.61)	3.73 (0.67 - 20.74)	
No	7	1363 (23.38)	104 (8.39)	1	
Hygienic precautions by veterinarian					0.04
No	18	4175 (71.62)	1020 (82.32)	7.00 (1.07 - 45.80)	
Yes	6	1654 (28.38)	219 (17.68)	1	

<sup>†</sup>Odds ratio calculated per 50 units change in measurement

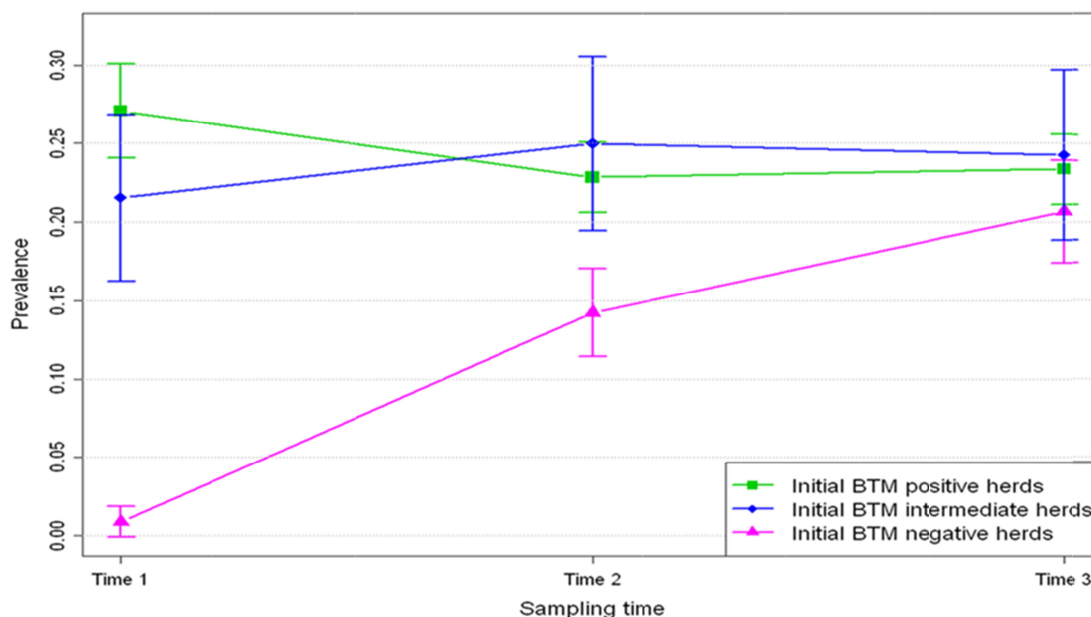
\*Odds ratio calculated per unit change in measurement

\*\*CI, confidence interval

\*\*\*Variables significant at  $p \leq 0.25$  were included in the table

The results of the multivariable logistic regression analysis accounting for random effect of herd and of cow are shown in Table 3. Ten variables remained in the final model: 1) breed, 2) parity, 3) milk yield, 4) protein content, 5) fat content, 6) season, 7) herd size, 8) types of stable, 9) quarantine, and 10) hygienic precautions taken by the veterinarian before entering the stable. A ROC curve with AUC as an indication of the predictive performance of the final model illustrated that the predictive ability of this model to be 0.79 (AUC=0.79). In the final model, 53% of the total variation was explained by variation between the herds ( $ICC_{\text{herd}}=0.53$ ) and 20% of the total variation was due to the variation between the cows ( $ICC_{\text{cow}}=0.20$ ).

## Estimation of prevalence



**Figure 2:** Cow prevalences of *Coxiella burnetii* antibody positivity at three time points in three strata of initially bulk tank milk (BTM) antibody positive, initially BTM antibody negative and initially BTM antibody intermediate herds in a prospective cross sectional study with follow-up conducted from August 2008 to June 2009 involving 3116 cows from 24 Danish dairy herds.

During the first sampling period the seroprevalence in initially BTM positive herds was 0.27 (95% CI [0.24 – 0.30]) and it varied significantly ( $P < 0.01$ ) from the prevalence of 0.009 (95% CI [0.00 – 0.01]) in herds which were initially BTM negative and also from the prevalence of 0.23 (95% CI [0.16 – 0.27]) in initially BTM intermediate herds. Significant variation of prevalence across the three strata was also found in the second sampling period but no notable difference was observed during the third sampling period (Figure 2). Prevalence in initially BTM negative herds increased significantly ( $P < 0.01$ ) from Time1 (0.009; 95% CI [0.00 – 0.01]) to Time3 (0.21; 95% CI [0.17 – 0.24]). There was a slight reduction of prevalence in initially BTM positive herds from Time1 to Time3 which was not statistically significant. However, the prevalence of positive cows in initially BTM intermediate herds was almost constant across the time periods (Figure 2).

## Discussion

This study confirms that breed and season are associated with the presence of *C. burnetii* antibodies in cattle as already known from previous studies (McCaughy et al., 2010; Cantas et al., 2011). In addition to these, we found that parity (i.e. exposure time), decreasing milk yield, increased protein content in milk, decreased fat content in milk and herd size, lack of quarantine of newly purchased animals, loose housing system and reduced hygienic precautions taken by the veterinarian before entering into the stable were significantly associated with the level of *C. burnetii* antibodies in milk. This study did not



identify an association between SCC and level of *C. burnetii* antibodies whereas Barlow et al. (2008) demonstrated a positive association between SCC and *C. burnetii* infection (detected by PCR) in a multivariable generalized linear model. These variations may not only be due to the different outcome variables modeled but also the different modeling approaches used. In the multivariable model we also did not find any association between reproductive problems and *C. burnetii* seropositivity in cows in contrast to the observations by Bildfell et al. (2000) and Khalili et al. (2011). However, these two studies only used univariable analysis and thus did not adjust for covariates.

**Table 3:** Results of the multivariable logistic regression analysis of cow and herd level factors associated with *Coxiella burnetii* antibody positivity in cow milk samples accounting for random effects of herd and of cow in a prospective cross sectional study with follow-up conducted from August 2008 to June 2009 involving 3116 cows from 24 Danish dairy herds.

Variables	Odds Ratio (95% CI)**	P
<b>Cow level factors</b>		
Breed		<0.01
<i>Danish Holstein</i>	3.21 (2.17 - 4.74)	
<i>Other</i>	2.92 (1.86 - 4.58)	
<i>Jersey</i>	1	
Parity		0.02
≥ 4	1.40 (1.11 - 1.77)	
3	1.27 (1.02 - 1.58)	
2	1.08 (0.90 - 1.29)	
1		
Milk yield (kg) <sup>†</sup>	0.98 (0.97 - 0.99)	<0.01
Fat content (%) <sup>†</sup>	0.88 (0.79 - 0.98)	0.02
Protein content (%) <sup>†</sup>	1.43 (1.22 - 1.69)	<0.01
Season		<0.01
<i>Summer</i>	1.55 (1.31 - 1.82)	
<i>Winter</i>	1.27 (1.08 - 1.50)	
<i>Autumn</i>	1	
<b>Herd level factors</b>		
Herd size*	1.75 (1.03 - 3.00)	0.04
Type of stable		
<i>Loose</i>	4.22 (1.08 - 16.57)	0.03
<i>Tie stall</i>	1	
Quarantine of newly purchased animals		0.02
<i>No</i>	3.75 (1.19 - 11.86)	
<i>Yes</i>	1	
Hygienic precautions taken by veterinarian		<0.01
<i>No</i>	8.91 (2.00 - 22.23)	
<i>Yes</i>	1	

<sup>†</sup>Odds ratio calculated per unit change in measurement

\*Odds ratio calculated per 50 units change in measurement

\*\*CI, confidence interval

This study demonstrates that the risk of a cow being antibody positive varied among breeds with a higher risk in Danish Holstein cows than Jersey cows. Our findings agree with a previous study in Northern Ireland (McCaughey et al., 2010). However, the reason for this apparent variation in breed susceptibility is unknown. Genotypic variation among the breeds is a possible explanation which needs to be investigated in depth to resolve this question.

The significant positive associations between the level of *C. burnetii* antibodies and the time duration of a possible exposure to *C. burnetii* were revealed by several time measurements such as cow age, parity and number of days a cow had stayed in the cow barn. Due to multicollinearity only the variable 'parity' was used in the multivariable model. Older cows had stayed in the cow barn for longer time than young animals. Hence the probability of being exposed to the bacterium, if present, increases with exposure time and therefore, it is not surprising that the *C. burnetii* antibody positivity increases with age. This result is in agreement with previous studies by McCaughey et al. (2010), Bottcher et al. (2011) and Garcia-Isperto et al. (2011).

This study revealed that seropositivity decreased with increasing milk yield contrary to findings of Garcia-Isperto et al. (2011). We found a positive association of *C. burnetii* antibody positivity with increasing days in milk. A high positive correlation between the *C. burnetii* infection status and cow days in milk was also reported by Barlow et al. (2008). However, this finding did not correspond to the findings of Rodolakis et al. (2007). The question which remained unanswered was whether this positive relation was due to the cows spending more time in cow barn and thus being more exposed to the bacterium or whether there was any immunogenic change during a lactation period which caused an increase in the antibody titre in milk. In the multivariable model we found that positivity increased with increasing level of milk raw protein content also including the immunoglobulins and with decreasing milk raw fat contents. The finding of a positive association with milk protein contents contradicted the findings of a previous study (Rose et al., 1994); however, it was unclear whether that study included measurement of immunoglobulins.

The present study demonstrated a seasonal variation in the occurrence of *C. burnetii* infection in Danish dairy cows with a higher risk in the summer. Seasonality in the occurrence of *C. burnetii* infection has been reported in cattle in Cyprus and Japan (Yanase et al., 1997; Cantas et al., 2011). Seasonal variation in occurrence of Q fever in humans has been reported by several authors. In most of the studies incidence was higher in spring and early summer (Tissot-Dupont et al., 1999; Hellenbrand et al., 2001).

In the present study seropositivity increased with increasing herd size. The positive association of herd size with *C. burnetii* infection in cattle was also reported by McCaughey et al. (2010) and Ryan et al. (2011). This observation may be explained by increased transmission probability with increasing number of cows in a herd. We also found that cows in loose housing systems had higher probability of being positive than cows housed in tie stall system. Loose housing system probably gives more random contact between uninfected and infected animals. Hence, risk of transmission probably increased in loose housing systems. It might also be due to indirect transmission from random

contamination and contact with the barn environment. Variation in the occurrence of *C. burnetii* infection in cattle among different housing types was also reported by Capuano et al. (2001).

Seropositivity in cows seemed to be lower in herds i) where quarantine was practiced for newly purchased animals, and ii) where veterinarians took adequate hygienic precautions (washing hands and changing cloths and boots before entering the barn). In addition, risk of being seropositive was found to be higher in cows from herds i) where cows came into contact with professional hoof trimmers and ii) where a maternity pen was used during parturition in univariable analyses. A recent study reported that purchase of animals increased the risk of introducing *C. burnetii* infection into cattle herds (EFSA Panel on Animal Health and Welfare (AHAW), 2010). This finding correlated with the biological assumption and stressed the importance of quarantine practice. Herd veterinarians and professional hoof trimmers might act as mechanical carriers and transfer the pathogen from infected to non-infected herds. This assumption was supported by a review article by Woldehiwet (2004) suggesting that farm personnel often act as mechanical transmitters of contaminated fomites from an infected herd to uninfected ones. Previous studies reported that risk of shedding *C. burnetii* bacteria increased during the calving period (To et al., 1998; Tissot-Dupont et al., 1999), with a consequent contamination of the calving area, e.g. the maternity pen. The infection might be transmitted to subsequent pregnant cows sharing the same maternity pen at parturition.

In our modelling approach we found significant clustering effects of herd and of cow. The final model (Table 3) showed a significant discriminatory ability (AUC=0.79) to classify seropositive and seronegative cows correctly. Our study included a relatively large number of cows and we selected most of the explanatory variables carefully assuming their plausible association with *C. burnetii* infection in cattle. The reason for including fat and protein contents in the analyses is that we used the milk yield without adjustment for energy. In the final multivariable model (Table 3) the inclusion of fat content only changed the estimates five percent for breed, and thus, not indicating a confounding effect of breed. In this study, 24 herds were selected following a stratified random sampling approach from 100 randomly selected herds for a prevalence study. All lactating cows within the selected herds were included. Thus, the study sample of 3116 cows represented 0.6 percent of the population of 560000 dairy cows. Post study sample size calculations for prevalence estimation (prevalence=0.20 ; allowable error=0.05) revealed the need of 255 cows, and the necessary sample size for a relative risk of 1.5 for infection in Holsteins versus Jersey cows (see data in Table 1) is 392 cows in each group. We met these requirements in the study. We therefore believe that the sample was sufficiently big and, due to random sampling, also representative of the population and without selection bias.

Our estimates of stratum specific (BTM antibody level) cow seroprevalences showed no significant changes across the time in herds which were initially BTM positive and BTM intermediate (Figure 2). Based on this finding we might conclude that if introduced once, *C. burnetii* infection can remain within a population for a long time i.e. at least for 11 months. Long persistence of *C. burnetii* infection in animals was also reported by Lang (1990). A significant increase in cow prevalence was observed in herds which were initially BTM negative. However, a careful evaluation showed that an increased cow prevalence was only seen in two of the ten initially negative herds, with an increase from almost zero to

about 60% during the study period (data not shown). Beyond the identified risk factors we could not explain this sudden change in prevalence.

The diagnostic test might introduce misclassification bias as it is neither 100% sensitive nor 100% specific. Horigan et al. (2011) reported high sensitivity (81.3%) and specificity (93.9%) of ELISA in cattle and the test has recently been used widely for the diagnosis of *C. burnetii* infection in cattle (Guatteo et al., 2011). Animal level information is recorded and updated routinely in the Danish Cattle Database. Disease information is dependent on a mandatory reporting after each visit by the herd veterinarian, which might introduce misclassification bias. We followed a standardized questionnaire at the telephone interviews with the farmers by reading the questions exactly as stated in the questionnaire in order to reduce misclassification, although recall bias due to farmers' lack of memory can not be ruled out. However, the procedure followed is standard in veterinary epidemiological studies. The quality of questionnaire data was validated on only one parameter, namely by comparing farmers' statements about purchase of animals with the information in the Danish Cattle Database. There was complete agreement on that variable.

## Conclusion

The individual animal factors like breed, days in cow barn, milk yield, milk protein contents, and the herd level factors like herd size, stable type, use of maternity pen, biosecurity related factors and season were found to have significant associations with the level of *C. burnetii* antibodies in cow milk samples. Infection with *C. burnetii* in herds may last quite long and seroconversion of BTM antibody status may occur due to the presence of a few latently infected cows or due to transmission from other herds. The results are important epidemiological contributions to our knowledge of the presence of *C. burnetii* antibodies.

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## **CHAPTER 5**

# **EVALUATION OF THE PERFORMANCE OF DIAGNOSTIC TESTS**





## CHAPTER 5

### **BAYESIAN ESTIMATION OF SENSITIVITY AND SPECIFICITY OF *COXIELLA BURNETII* ANTIBODY ELISA TESTS IN BOVINE BLOOD AND MILK**

**Suman Paul<sup>a</sup>**, Nils Toft<sup>a</sup>, Jørgen S. Agerholm<sup>a</sup>, Anna-Bodil Christoffersen<sup>b</sup> , Jens F. Agger<sup>a</sup>

<sup>a</sup> Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

<sup>b</sup> National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.

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## Abstract

Serological tests for *Coxiella burnetii* (the causative agent of Q fever) antibodies are usually based on enzyme linked immunosorbent assay (ELISA) although this method is not thoroughly evaluated. The objective of this study was to determine the sensitivity and specificity of an ELISA for detection of *C. burnetii* antibodies in milk and blood samples, using latent class models in a Bayesian analysis. Blood and milk samples of 568 lactating cows from 17 Danish dairy cattle herds collected in 2008 were used.

The best combination of sensitivity and specificity estimates was revealed at a sample to positive (S/P) cut-off of 40 for both blood and milk ELISAs. At this cut-off, sensitivity of milk ELISA was 0.86 (95% posterior credibility interval [PCI] [0.76; 0.96]). This was slightly but insignificantly higher than sensitivity of blood ELISA (0.84; 95% PCI [0.75; 0.93]). The specificity estimates of the ELISA methods on milk and blood were equal to 0.99. No conditional dependence was observed between the specificity estimates of the two test methods. However, the sensitivity estimates of both tests were significantly reduced when conditional covariances  $\geq 40$  were used. Collection of milk samples from lactating cows is relatively easy, non-invasive and inexpensive and hence milk ELISA may be a better option for screening lactating cows. But, blood ELISA is an option for screening non-lactating cattle.

**Key words:** *Coxiella burnetii*; ELISA; sensitivity and specificity; conditional dependence; Bayesian analysis; cattle.

## Introduction

Diagnosis of *Coxiella burnetii* infection (Q fever) in animals depends on detection of bacteria, bacterial components or antibodies (Bouvery et al., 2003; Fournier et al., 1998; Rodolakis, 2006). Detection of bacteria can be done by culturing, but usually detection of bacterial components, e.g. by PCR is used (Rodolakis, 2006). Although PCR is often considered the most appropriate technique for *C. burnetii* detection, it is an expensive and time consuming procedure and it depends on the actual presence of bacterial DNA (Rousset et al., 2010). Therefore, cheap and fast serological techniques are still widely used as screening tests and in large scale epidemiological studies. However, serological tests can only identify the antibody response to infection. CFT and ELISA are the two most commonly used serological techniques for screening in animals for *C. burnetii* exposure (Rousset et al., 2010). Nowadays, ELISA is becoming more popular than CFT for its better reliability and handiness (Rousset et al., 2010) and a recommended test for Q fever diagnosis in the European Union (EFSA Panel on Animal Health and Welfare (AHAW), 2010). ELISA can be used to detect antibodies both in milk and in blood samples. In recent years, milk analyses have been widely used in epidemiological studies involving dairy cattle since they have some advantages over blood analyses. Milk samples are easier and less expensive to collect, and non-invasive and hence minimize stress to the cow. However, blood ELISA is still necessary for the studies involving non-lactating cattle.

The performance of a diagnostic serological test can be evaluated against a perfect test with 100% sensitivity and 100% specificity (gold standard). However, none of the existing *C. burnetii* serological tests are perfect, as also known for diagnostic tests of most other infections (Rousset et al., 2010). Performance of the *C. burnetii* antibody ELISA has been evaluated in few studies based on the performance of CFT, which gave relative estimates of sensitivity and specificity (Emery et al., 2012; Kittelberger et al., 2009). In situations where the case definition is determined by an imperfect reference test, selection bias and/or misclassification bias might be introduced (Nielsen and Toft, 2002). In a situation where a reference test is unavailable, latent class analysis (LCA) is an alternative option for estimating sensitivity and specificity of two or more tests without assuming the underlying true antibody status of test subjects, which eliminates the chance of selection bias in the estimates. LCA can be done either by fitting the model using maximum likelihood procedures or by Bayesian analysis (Enøe et al., 2000). In LCA, three assumptions known as the Hui-Walter paradigm are generally made: (1) at least two populations with different prevalences are required, (2) the sensitivity and specificity of the tests do not differ across the populations, and (3) the tests under evaluation are conditionally independent given the disease status (Hui and Walter, 1980). However, especially for the tests based on the same biological principle, in our case detection of antibodies, the assumption about conditional independence given disease status can be questioned. In a recent study the performance of CFT and three commercially available ELISAs were evaluated in a LCA framework by fitting the model using maximum likelihood estimation (Horigan et al., 2011). In that study all the tests were assumed to be conditionally independent. This assumption of conditional independence influences the test properties. If a positive correlation between the tests under evaluation is ignored it will lead to overestimation of the test performance, while ignorance of a negative correlation will cause underestimation of the test performance (Vacek, 1985).

The objective of this study was to estimate the sensitivity and specificity of milk ELISA and blood ELISA for detection of *C. burnetii* antibodies accounting for the conditional dependence between the two test methods, using latent class models in a Bayesian analysis in a population of spontaneously infected cows from selected Danish dairy cattle herds.

## **Materials and methods**

### **Sample collection and population stratification**

The data included 568 lactating cows from 17 Danish dairy cattle herds. The herds were recruited from 100 dairy herds that were randomly selected among 4785 milk delivering herds in the spring 2008 and examined for the level of *C. burnetii* antibodies in a bulk tank milk (BTM) sample (Agger et al., 2010). The 100 herds were stratified into positive, negative and intermediate herds based on the level of *C. burnetii* antibodies (expressed as S/P value) in a single BTM sample. Based on the cut-off recommended by the manufacturer, a herd with  $S/P \geq 40$  was considered positive and a herd with  $S/P < 30$  was negative, whereas a herd with  $S/P$  30-39 was classified as intermediate. Following this initial study, eight positive, six negative and three intermediate herds were selected by systematic random sampling within the three strata. These three groups were used to form the subpopulations with assumed differences in animal-level prevalences. Milk of all lactating cows within the selected herds were sampled. However, only 568 of these were blood sampled due to the economics of the project. Only cows with both sample types were included in this study, i.e. 568 cows. The cows to be blood sampled were selected by a within herd systematic random sampling procedure. Milk sampling was done by the farmers as part of the milk control scheme and the blood samples were taken by the herd veterinarians. The time interval between the milk and blood sampling was minimized and ranged from zero to three days with median one. All samples were collected between August 2008 and October 2008 and sent to the National Veterinary Institute, Technical University of Denmark maintaining standard cool chain and tested by ELISA according to instructions by the test kit manufacturer.

### **Diagnostic tests**

#### ***Milk ELISA***

Ten milliliter milk from each cow was collected for testing. The fat fraction was removed from the milk by centrifugation and the non-fat fraction was stored at  $-20^{\circ}\text{C}$  until tested for antibodies against *C. burnetii* using the commercial CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland) based on *C. burnetii* inactivated phase 1 and phase 2 antigens. All samples were tested in duplicates and the optical densities (OD) of the samples were averaged and corrected by subtracting the OD of the negative control. The results were expressed as S/P values and estimated using the equation recommended by the manufacturer (Agger et al. 2010; Paul et al. 2012). The S/P values were estimated on a continuous scale with a theoretical range from zero to “plus infinity”. The manufacturer of the test kit recommends a S/P cut-off  $\geq 40$  for classifying test subjects as positive. At this cut-off we considered a sample with  $S/P < 40$  as negative. However, according to our study objective we investigated the test results according to

three different S/P cut-offs where S/P values  $\geq 30$ ,  $\geq 40$  and  $\geq 50$ , respectively were considered as test positive, and S/P values  $< 30$ ,  $< 40$  and  $< 50$  were considered test negative, respectively.

### **Blood ELISA**

Five to eight milliliter blood from each selected cow was collected. Upon arrival at the laboratory, samples were centrifuged at  $3000 \times g$  for 10 minutes for serum separation. Sera were stored at  $5^\circ C$  and tested for antibodies against *C. burnetii*. The same ELISA kit and laboratory procedure was used for milk and blood samples and the results were expressed as S/P values with cut-off values as mentioned above.

### **Target condition**

The target condition of this analysis was considered to be cows that had humoral antibodies to *C. burnetii*, i.e. had an immunological response. This implies an underlying latent condition, where antibodies are present in the cow although not necessarily at a detectable level in either blood or milk.

### **Statistical model**

Initially we estimated the sensitivity and specificity of milk and blood ELISAs using a LCA using a conditionally independent model (CID model) (Branscum et al., 2005; Mweu et al., 2012; Toft et al., 2007a). We assumed that the within herd true prevalence of *C. burnetii* antibody positivity differed across the three populations. Additionally, the sensitivity and specificity of each of the two diagnostic tests were modeled to be constant across the populations. This model yields nine degrees of freedom (three from each population) which are sufficient to estimate seven parameters (sensitivity and specificity of each test and prevalences of three populations). A Bayesian model in OpenBUGS version 3.2.1 rev 781 (Thomas et al., 2006) was implemented to estimate the test parameters and population prevalences.

To evaluate the assumption of conditional independence we compared the estimates of the CID model with the estimates of a model which assumed dependency of the two tests (COC model). We ran these COC models using a covariance expressed as a proportion (e.g., 0.00, 0.10, 0.20, 0.25, 0.30, 0.40, 0.50 and 0.75) of the maximum conditional covariance  $Se_B$  ( $\lambda_{Se}$  and  $\lambda_{Sp}$ ). The upper and lower limits of  $\lambda_{Se}$  and  $\lambda_{Sp}$  can be derived from the probabilities in **Prob<sub>i</sub>** since these must be between zero and one (Toft et al., 2007a):

$$L\lambda_{Se} = \max [-(1-Se_M)(1-Se_B), -Se_M Se_B] \leq \lambda_{Se} \leq U\lambda_{Se} = \min [Se_M (1-Se_B), Se_B (1-Se_M)] \quad (1)$$

$$L\lambda_{Sp} = \max [-(1-Sp_M)(1-Sp_B), -Sp_M Sp_B] \leq \lambda_{Sp} \leq U\lambda_{Sp} = \min [Sp_M (1-Sp_B), Sp_B (1-Sp_M)] \quad (2)$$

where  $Se_M$  and  $Se_B$  are the sensitivity of milk ELISA and blood ELISA, respectively. The same applies for specificity. L and U are the upper and lower limit of  $\lambda_{Se}$  and  $\lambda_{Sp}$ . Fixing the conditional covariances (using the approach from Vacek, 1985) allowed us to estimate the remaining seven parameters of the model:

$$\begin{aligned}
\text{Prob}_i &= \begin{pmatrix} \Pr(T_1^+ T_2^+) \\ \Pr(T_1^+ T_2^-) \\ \Pr(T_1^- T_2^+) \\ \Pr(T_1^- T_2^-) \end{pmatrix} = \begin{pmatrix} \Pr(T_1^+ T_2^+ | D^+) \Pr(D^+) + \Pr(T_1^+ T_2^+ | D^-) \Pr(D^-) \\ \Pr(T_1^+ T_2^- | D^+) \Pr(D^+) + \Pr(T_1^+ T_2^- | D^-) \Pr(D^-) \\ \Pr(T_1^- T_2^+ | D^+) \Pr(D^+) + \Pr(T_1^- T_2^+ | D^-) \Pr(D^-) \\ \Pr(T_1^- T_2^- | D^+) \Pr(D^+) + \Pr(T_1^- T_2^- | D^-) \Pr(D^-) \end{pmatrix} \\
&= \begin{pmatrix} (\text{Se}_M \text{Se}_B + U\lambda_{\text{Se}}) p_i + ((1 - \text{Sp}_M)(1 - \text{Sp}_B) + U\lambda_{\text{Sp}})(1 - p_i) \\ (\text{Se}_M(1 - \text{Se}_B) - U\lambda_{\text{Se}}) p_i + ((1 - \text{Sp}_M)\text{Sp}_B - U\lambda_{\text{Sp}})(1 - p_i) \\ ((1 - \text{Se}_M)\text{Se}_B - U\lambda_{\text{Se}}) p_i + (\text{Sp}_M(1 - \text{Sp}_B) - U\lambda_{\text{Sp}})(1 - p_i) \\ ((1 - \text{Se}_M)(1 - \text{Se}_B) + U\lambda_{\text{Se}}) p_i + (\text{Sp}_M \text{Sp}_B + U\lambda_{\text{Sp}})(1 - p_i) \end{pmatrix} \quad (3)
\end{aligned}$$

where  $U\lambda_{\text{Se}}$  and  $U\lambda_{\text{Sp}}$  are the fixed proportion of upper limit of conditional covariances for the sensitivities and specificities, respectively. For  $U\lambda_{\text{Se}}$  and  $U\lambda_{\text{Sp}} = 0.00, 0.10, 0.20, 0.25, 0.30, 0.40, 0.50$  and  $0.75$ . We have only used the  $U\lambda_{\text{Se}}$  and  $U\lambda_{\text{Sp}}$  in the model as we believe the two test methods are positively correlated, if correlated at all. This model will be the CID model if we consider  $U\lambda_{\text{Se}}$  and  $U\lambda_{\text{Sp}} = 0$ . Estimating the sensitivity and specificity at different predefined fixed proportions of conditional covariances allowed us to evaluate the effect of conditional dependence of these two tests on the parameter estimates.

All models in this study were identifiable. Therefore, we used uninformative priors (beta (1,1)) for sensitivity and specificity of both tests and also for the prevalences in three populations. For all analyses, we ran 20,000 iterations of the models with the first 10,000 discarded as the burn-in phase. To assess convergence of the Markov Chain Monte Carlo (MCMC) chain we visually inspected the time-series plots of selected variables as well as the Gelman-Rubin diagnostic plots using three sample chains with different initial values (Toft et al., 2007b).

The hypotheses of the differences between test parameters were tested by calculating the Bayesian posterior probabilities (POPR) for e.g.  $H_0: \text{Se}_M > \text{Se}_B$ . The deviance information criterion (DIC) statistics of the resulting models were compared to assess the goodness of fit (the smaller the value the better the fit). We also computed the differential positive rate ( $\text{DPR} = (\text{Se} + \text{Sp}) - 1$ ), which indicates the cut-off value at which the sensitivity and specificity are maximized simultaneously. Selection of cut-off for diagnostic tests based on DPR implies an equal misclassification cost of false positive and false negative interpretations (Greiner et al., 2000).

## Results

There were 362 (63.73%), 141 (24.82%) and 65 (11.44%) cows included in the eight positive, the six negative and the three intermediate herds, respectively. The cross-tabulated distribution of the dichotomous outcome of the two tests at cut-off 40 is shown in Table 1.

Based on the data shown in Table 1, the sensitivity and specificity of the two tests and three population-specific prevalences were estimated using two different types of models; one assuming CID between the two tests given serology status, and one assuming COC between the two tests. Results of these two methods are shown in Table 2. Comparison of the results of these methods shows that the sensitivity of the two tests changed



significantly only for  $\text{COC} \geq 0.40$  and no visible changes and differences was observed in the specificity estimates of the two tests (Table 2). Remarkable changes in the population-specific prevalence estimates for positive and intermediate herds were also seen in the models with  $\text{COC} \geq 0.40$  (Table 2). The CID model has the smallest DIC among the resulting models suggesting the CID model as the preferred one (Table 2).

**Table 1:** Cross-tabulated results for milk and blood ELISA at cut-off S/P= 40 for 568 cows stratified by herd status.

<b>Herd status</b>	<b>POS<sub>M</sub>/POS<sub>B</sub><sup>a</sup></b>	<b>POS<sub>M</sub>/NEG<sub>B</sub><sup>b</sup></b>	<b>NEG<sub>M</sub>/POS<sub>B</sub></b>	<b>NEG<sub>M</sub>/NEG<sub>B</sub></b>	<b>Total</b>
<b>Positive</b>	76	17	14	255	<b>362</b>
<b>Intermediate</b>	6	1	3	55	<b>65</b>
<b>Negative</b>	0	1	1	139	<b>141</b>
<b>Total</b>	<b>82</b>	<b>19</b>	<b>18</b>	<b>449</b>	<b>568</b>

M: milk; B: blood

<sup>a</sup> Positive

<sup>b</sup> Negative

DPR estimates of the CID models confirmed that both tests performed better at cut-off S/P = 40 than at any other cut-offs (Table 3). At this cut-off the sensitivity of the milk ELISA was slightly higher than the sensitivity of the blood ELISA. However, this variation of estimates was not statistically significant. The specificity estimates of the two tests at this cut-off were similar (Table 3). No improvement in the sensitivity and specificity estimates of two tests was observed when the cut-off was reduced to S/P = 30, but the sensitivity estimates decreased for both tests when cut-off S/P = 50 was used.

## Discussion

We estimated the sensitivity and specificity of an ELISA applied to blood and milk samples of dairy cows using latent class analysis to avoid the implications of an assumed perfect reference test. The analysis showed that both tests are equally highly specific whereas milk ELISA has marginally higher sensitivity than the blood ELISA. However, this difference of sensitivity estimates is of negligible practical importance. The sensitivity and specificity estimates of blood ELISA are comparable with estimates of the study of Horigan et al. (2011) but disagree with the relative estimates reported by Kittelberger et al. (2009). In the latter study an ELISA was compared with the imperfect reference CFT, which defined the cases and might have influenced the parameter estimates of the test under evaluation (Nielsen and Toft, 2002). To the best of our knowledge, there are no recent studies that report the sensitivity and specificity of milk ELISA in individual cows. However, a high agreement (Kappa=0.89) between blood and milk ELISA was reported by Guatteo et al. (2007).

A previous study showed that the apparent prevalences of *C. burnetii* seropositivity within the three strata used in this analysis varied significantly (Paul et al., 2012). Posterior estimates of stratum specific prevalences in this study were also very different, which support that the stratification of the population into subpopulations with differences in prevalence was successful. The assumption of consistency in sensitivity and specificity estimates across the population was difficult to evaluate in the current study. However, in a separate analysis considering negative and intermediate herds as a single population we

**Table 2:** The mean estimates and 95% posterior credibility intervals (PCI) of the sensitivity (Se) and specificity (Sp) of milk and blood ELISA and population specific prevalences of the conditionally independent (CID) model and conditionally dependent (COC) models assuming different proportions of maximum conditional covariances.

Conditional covariance <sup>a</sup>	Tests characteristics (95% PCI)				Prevalences			DIC <sup>b</sup>
	Se <sub>M</sub>	Sp <sub>M</sub>	Se <sub>B</sub>	Sp <sub>B</sub>	POS	INT	NEG	
CID model								
0.00	0.86 (0.76;0.96) <sup>c</sup>	0.99 (0.97;1.00)	0.84 (0.75;0.93) <sup>d</sup>	0.99 (0.96;1.00)	0.28 (0.23;0.34)	0.14 (0.06;0.25)	0.01 (0.00; 0.03)	41.55
COC models								
0.10	0.86 (0.76;0.97)	0.99 (0.97;1.00)	0.84 (0.74;0.93)	0.99 (0.96;1.00)	0.28 (0.23; 0.34)	0.14 (0.06; 0.25)	0.01 (0.00; 0.03)	41.99
0.20	0.84 (0.74;0.96)	0.99 (0.97;1.00)	0.82 (0.73;0.92)	0.99 (0.96;1.00)	0.29 (0.23; 0.35)	0.14 (0.06; 0.25)	0.01 (0.00; 0.03)	42.10
0.25	0.83 (0.73;0.95)	0.99 (0.97;1.00)	0.81 (0.72;0.92)	0.99 (0.96;1.00)	0.29 (0.23; 0.35)	0.14 (0.06; 0.25)	0.01 (0.00; 0.03)	42.54
0.30	0.83 (0.72;0.95)	0.99 (0.97;1.00)	0.81 (0.71;0.91)	0.99 (0.96;1.00)	0.30 (0.24; 0.35)	0.14 (0.06; 0.26)	0.01 (0.00; 0.04)	42.79
0.40	0.76 (0.63;0.92)	0.99 (0.96;1.00)	0.74 (0.62;0.87)	0.99 (0.96;1.00)	0.32 (0.25; 0.40)	0.16 (0.06; 0.28)	0.01 (0.00; 0.04)	43.16
0.50	0.70 (0.54;0.90)	0.99 (0.96;1.00)	0.69 (0.54;0.84)	0.99 (0.96;1.00)	0.35 (0.26; 0.45)	0.17 (0.07; 0.31)	0.01 (0.00; 0.04)	43.36
0.75	0.36 (0.24;0.72)	0.99 (0.96;1.00)	0.36 (0.24;0.66)	0.98 (0.95;1.00)	0.68 (0.34; 0.98)	0.33 (0.11; 0.70)	0.01 (0.00; 0.09)	51.15

M: milk ELISA; B: blood ELISA; POS: positive herds; INT: intermediate herds; NEG: negative herd

<sup>a</sup> Proportion of maximum upper limit of conditional covariance

<sup>b</sup> Deviance information criterion

<sup>c</sup> Statistically insignificant difference

<sup>d</sup> Statistically insignificant difference

**Table 3:** The mean estimates and 95% PCI of the sensitivity (Se) and specificity (Sp) of milk and blood ELISA, and population specific prevalences of the conditionally independent (CID) models at different cut-offs.

Test parameter	Estimates (95% PCI)	DPR <sub>M</sub> <sup>a</sup>	DPR <sub>B</sub>
<i>Cut-off 30</i>			
<b>Se<sub>M</sub></b>	0.86 (0.76; 0.96)	0.85	0.82
<b>Sp<sub>M</sub></b>	0.99 (0.97; 1.00)		
<b>Se<sub>B</sub></b>	0.84 (0.75; 0.92)		
<b>Sp<sub>B</sub></b>	0.98 (0.96; 1.00)		
<b>P<sub>POS</sub></b>	0.29 (0.24; 0.36)		
<b>P<sub>INT</sub></b>	0.16 (0.08; 0.27)		
<b>P<sub>NEG</sub></b>	0.02 (0.01; 0.06)		
<i>Cut-off 40</i>			
<b>Se<sub>M</sub></b>	0.86 (0.76; 0.96)	0.85	0.83
<b>Sp<sub>M</sub></b>	0.99 (0.97; 1.00)		
<b>Se<sub>B</sub></b>	0.84 (0.75; 0.93)		
<b>Sp<sub>B</sub></b>	0.99 (0.96; 1.00)		
<b>P<sub>POS</sub></b>	0.28 (0.23; 0.34)		
<b>P<sub>INT</sub></b>	0.14 (0.06; 0.25)		
<b>P<sub>NEG</sub></b>	0.01 (0.00; 0.03)		
<i>Cut-off 50</i>			
<b>Se<sub>M</sub></b>	0.82 (0.73; 0.91)	0.81	0.81
<b>Sp<sub>M</sub></b>	0.99 (0.97; 1.00)		
<b>Se<sub>B</sub></b>	0.82 (0.73; 0.92)		
<b>Sp<sub>B</sub></b>	0.99 (0.98; 1.00)		
<b>P<sub>POS</sub></b>	0.28 (0.23; 0.33)		
<b>P<sub>INT</sub></b>	0.12 (0.04; 0.19)		
<b>P<sub>NEG</sub></b>	0.01 (0.00; 0.03)		

P: prevalence; POS: positive herds; INT: intermediate herds; NEG: negative herds

<sup>a</sup> DPR: (Se+Sp-1)

did not find any changes in the sensitivity and specificity estimates (results not shown). We therefore believe that the assumptions of constant sensitivity and specificity across the populations were justified. Toft et al. (2005) demonstrated that difference in population prevalences can influence the sensitivity estimate of the tests and estimates increase with increasing population prevalence. However, in our case changes in population prevalence did not change the sensitivity estimates of the tests.

The assumption of conditional independence between the two tests was questionable since it is the same test applied to two different media. These two tests are supposed to be correlated given antibody status, unless the antibody definition is selected to reflect this. We accounted for this correlation in the COC models to avoid the overestimation or underestimation of the performance of the tests (Vacek, 1985). Our analyses showed that the specificity estimates of the tests did not change even when a high proportion of

conditional covariance (0.75) was used. Significant reductions in the sensitivity and prevalence estimates were observed when the proportion of conditional covariance exceeded 0.40 (Table 2). The estimates of a COC model without fixing the maximum limit of the covariance parameters also suggest that there is no significant conditional dependence between the sensitivity and specificity of two ELISA methods (results not shown). Biologically it may not be true as both tests are based on the same biological principle. These two tests have very high sensitivity and almost perfect specificity. Therefore, the proportion of absolute dependence between these two tests might be very small and the estimates of COC models do not differ greatly from the estimates of CID models. It implies that the two tests might be conditionally dependent but this dependence does not affect sensitivity and specificity estimates when evaluated against each other. A positive correlation between tests could be expected if the tests are based on the same physiological phenomenon. In our case, both tests are based on the same antibody reaction; hence something which inhibits the reaction or causes a false reaction for one of the tests may have a similar effect on the other (Vacek, 1985). An increasing degree of correlation between two tests implies that this reaction is becoming more pronounced. In our study this is most notable in the sensitivity estimates, where an increase in the degree of correlation reduces the sensitivity of both tests, which then as a consequence forces the true prevalence estimates to increase. The latter is most notable in the populations where disease is most prevalent (Table 2).

As the sensitivity and specificity estimates of CID model and COC models with conditional covariance < 40 were almost similar the estimates of test parameters found in the CID model seem valid. We compared the test parameter estimates of the CID model using recommended cut-off with the models with two other cut-offs. Results showed that the sensitivity and the specificity estimates did not change when S/P= 30 was used as cut-off. On the other hand, the sensitivity estimate reduced slightly when S/P=50 was used as cut-off (Table 3). DPR estimates also confirmed that S/P = 40 was the best cut-off for both blood and milk ELISA. Therefore, cut-off at S/P = 40 can be recommended for ELISA for classifying the serological status of a cow. Both milk and blood ELISAs for detecting *C. burnetii* seropositive cows can provide false positive and false negative results as neither are analytically perfect at any cut-off. False positive interpretation may result from cross reaction with antibodies provoked by other pathogens. Antigens of *C. burnetii* for example, can cross-react with antibodies of *Bartonella spp.*, *Legionella spp.* and *Chlamydiae spp.* (Finidori et al., 1992; LaScola and Raoult, 1996; Lukacova et al., 1999; Musso and Raoult, 1997; Vermeulen et al., 2010; Wilson et al., 1992). On the other hand, a cow with recent infection during sampling time may not produce sufficient amount of antibodies that are detectable in both blood and milk yet. This might give false negative results (Greiner and Gardner, 2000).

In this analysis the target condition was reflecting an objective to identify seropositive cows as an indication of exposure to *C. burnetii* bacteria. *C. burnetii* infection has a long persistence and can evoke a long lasting antibody response (Garcia-Ispuerto et al., 2011; Lang, 1990). Estimation of test performance of serological tests using LCA is recommended for chronic, persistent diseases where an antibody response can be detectable for a long period (Branscum et al., 2005). Therefore, LCA seems like a viable approach for evaluating serological tests for *C. burnetii* infection.

## Conclusion

This LCA for estimating the sensitivity and the specificity of milk and blood ELISAs for detecting *C. burnetii* antibodies in Danish dairy cows showed that the sensitivity of milk ELISA (0.86) is almost similar to that of blood ELISA (0.84) at the cut-off S/P = 40. In addition, the specificity of milk ELISA (0.99) is equal to that of blood ELISA (0.99) at the same cut-off. Although both test methods have similar test characteristics, milk ELISA has some practical and less cumbersome advantages over blood ELISA. Collection of milk is non-invasive, easy and less expensive and less physically risky for the sampling person as compared to the collection of blood. Hence, milk ELISA as a screening test might be a better option for *C. burnetii* surveillance programs in lactating dairy cows.

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## **CHAPTER 6**

### **ADJUSTMENT OF DIAGNOSTIC TEST CHARACTERISTICS FOR ESTIMATING PREVALENCE AND RISK FACTORS OF *COXIELLA BURNETII* SEROPOSITIVITY IN SLAUGHTER CATTLE**





## CHAPTER 6

# **PREVALENCE AND RISK FACTORS OF *COXIELLA BURNETII* SEROPOSITIVITY IN DANISH BEEF AND DAIRY CATTLE AT SLAUGHTER, ADJUSTED FOR TEST UNCERTAINTY**

**Suman Paul<sup>a</sup>**, Jens F. Agger<sup>a</sup>, Jørgen S. Agerholm<sup>a</sup>, Bo Markussen<sup>b</sup>

a Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark.

b Laboratory of Applied Statistics, Department of Mathematical Sciences, Faculty of Science, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark.

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## Abstract

Antibodies to *Coxiella burnetii* have been found in the Danish dairy cattle population with high levels of herd and within herd seroprevalence. However, the prevalence of antibodies to *C. burnetii* in Danish beef cattle remains unknown. The objectives of this study were to (1) estimate the prevalence and (2) identify risk factors associated with *C. burnetii* seropositivity in beef and dairy cattle based on sampling at slaughter in Denmark.

Eight hundred blood samples from slaughtered cattle were collected from six Danish slaughter houses from August to October, 2012, following a random sampling procedure. Blood samples were tested by a commercially available *C. burnetii* antibody ELISA kit. A sample was defined as positive, if the sample-to-positive ratio was greater than or equal to 40. Animal and herd information were extracted from the Danish Cattle Database. Apparent (AP) and true prevalence (TP) were specific to the breed, breed groups, gender and herd type; and breed-specific true prevalence with a random effect of breed was estimated in a Bayesian framework. A Bayesian logistic regression model was used to identify risk factors of *C. burnetii* seropositivity. Test sensitivity and specificity estimates from a previous study involving Danish dairy cattle were used to generate prior information.

The prevalence was significantly higher in dairy breeds (AP=9.11%; TP=9.45%) than in beef breeds (AP=4.32%; TP=3.54%), in females (AP=9.10%; TP=9.40%) than in males (AP=3.62%; TP=2.61%) and in dairy herds (AP=15.10%; TP=16.67%) compared to beef herds (AP=4.54%; TP=3.66%). The Bayesian logistic regression model identified breed groups along with age and number of movements, as contributors for *C. burnetii* seropositivity. The risk of seropositivity increased with age and increasing number of movements between herds. Results indicate that seroprevalence of *C. burnetii* is lower in cattle sent for slaughter than in Danish dairy cows in production units. A greater proportion of this prevalence is attributed to slaughtered cattle of dairy breeds or cattle raised in dairy herds rather than beef breeds.

**Key words:** *Coxiella burnetii*; cattle; seroprevalence; true prevalence; test uncertainty; Bayesian analysis.

## Introduction

*Coxiella burnetii*, the causative agent of Q fever, is an obligate intracellular bacterium from the family *Coxiellaceae* (Angelakis and Raoult, 2010). This bacterium can infect a wide range of animals, but cattle, sheep and goats are the principal reservoirs and sources of human infection (Angelakis and Raoult, 2010). Infection in cattle usually remains unrecognised (Rodolakis, 2009), but it may cause sporadic reproductive problems such as abortion (Agerholm, 2013). Infected animals may shed large quantities of bacteria during parturition and in milk, faeces, urine and vaginal mucus for a long period (Guatteo et al., 2006; Rodolakis et al., 2007).

In 2008, bulk tank milk (BTM) samples of 100 randomly selected Danish dairy herds were tested to estimate the prevalence of *C. burnetii* antibody positivity (Agger et al., 2010). In that study, 59% of the herds tested positive at a cut-off of 40 sample-to-positive (S/P) ratio in the test used (milk antibody ELISA, Chekit-Q-fever, IDEXX, Liebefeld-Bern, Switzerland), 30% tested negative (S/P < 30) and 11% of the samples gave doubtful (intermediate) results (S/P 30-39). A within herd prevalence study carried out in the autumn of 2008 involving 24 herds from the previous study (Agger et al., 2010) showed that 27% , 23% and 0.9% of cows tested positive in BTM positive, intermediate and negative herds, respectively (Paul et al., 2012). However, knowledge about herd or animal levels of *C. burnetii* seroprevalence in Danish beef cattle is scarce. Danish beef cattle herds generally operate extensively and with a mean herd size of only 12 animals (Anonymous, 2012). Although there are some variations in Danish dairy and beef cattle production systems, these two populations are not completely separated. Thus, if *C. burnetii* infection is present in the beef cattle population, this might potentially be a source of infection for other domestic ruminants and humans.

In most of the survey or surveillance programs, a readily available serological test is used to test a group of animals to obtain an indirect measure of disease or exposure (e.g., apparent prevalence (AP)). This might provide an overview of the level of seropositive animals within a specific population. However, very few diagnostic tests appear to be perfect. Ignorance of test uncertainty may lead not only to misclassification of diseased (exposed) and non-diseased (non-exposed) animals, and biased estimates of measures of association in risk factor studies, but also to provide a higher level of confidence than the reality. These may negatively affect the surveillance and/or control and eradication program. Bayesian analysis provides a framework for adjustment for imperfections or the uncertainties of the diagnostic tests to obtain an unbiased estimate (e.g., true prevalence (TP)) (Tu et al., 1999). Similar adjustments for misclassifications can also be included in the logistic regression model in the Bayesian framework in the form of prior distribution. This will allow for an estimation of the effect of a risk factor on the true (latent) occurrence of the disease/infection (McInturff et al., 2004). Most prevalence surveys do not include adjustment for test uncertainty, due to lack of the reliable parameter estimates in the literature, or parameter estimates are only available for specific populations and locations (Greiner and Gardner, 2000).

Several evaluations of ELISA to detect *C. burnetii* antibodies have been reported in recent years. In some of these studies, serum ELISA was evaluated against a complement

fixation test (CFT), which gave relative estimates of sensitivity (Se) and specificity (Sp) (Emery et al., 2012; Kittelberger et al., 2009). Horigan et al. (2011) studied the performance of CFT and serum ELISAs in a latent class analysis (LCA) framework by fitting the model using maximum likelihood estimation. Paul et al. (2013) estimated the Se and Sp of milk and serum antibody ELISAs in a Bayesian framework, and found the Se (84%) and Sp (99%) of blood ELISA.

The objectives of this study were to (1) estimate the AP and TP of *C. burnetii* seropositivity and (2) identify the risk factors associated with *C. burnetii* seropositivity in cattle raised for meat production and sampled at slaughter in Denmark using the Bayesian framework where the uncertainty about the Se and Sp of the diagnostic test was accounted for.

## **Materials and methods**

### **Sample selection and diagnostic test**

Blood samples for the study were collected from slaughter cattle in the Danish surveillance programs for Bovine Virus Diarrhea Virus infection, Infectious Bovine Rhinotracheitis virus infection and Salmonella Dublin infection. According to these programs, blood is sampled from at least one slaughtered cattle per herd at least every fourth months, provided the herd sent animals to slaughter. The blood samples were tested at the Eurofins Steins Laboratorium A/S Denmark for antibodies to these infections, and the remaining serum of samples collected during August to October 2012 were used in this study and tested for *C. burnetii* antibodies.

The blood samples were only marked with the animal id-number, and therefore, it was not possible for the technician to differentiate between samples of beef cattle breeds, and samples of bulls of dairy breeds or dairy x beef cattle raised for meat production; and thus, we accounted for this in the sample size computation. The necessary sample size was estimated to 196 samples under the assumption of a maximum prevalence of test positivity of 15% in the sampling population, and an allowable error of 5% in a population of >50,000 slaughtered cattle. As cattle of both beef and dairy herd origins were selected, we decided to test 800 blood samples for antibodies of *C. burnetii*, and to account for the cattle type in the statistical analyses. At the slaughter houses, the surveillance system focused on cattle from non-dairy herds, as dairy herds are monitored on BTM samples. Therefore, our sample primarily contained cattle from non-dairy herds. However, most slaughter houses receive cattle of beef as well as dairy breeds, both males and females, and from several regions of Denmark. Therefore, few animals of dairy breeds (both male and female) in the study population originated from dairy herds, and we included them in the statistical analyses for comparative purposes. As the sampled cattle were randomly selected, we considered them representative of Danish slaughtered cattle.

The blood samples were tested for IgG antibodies to *C. burnetii* based on phase I and II antigens using a commercial antibody ELISA kit (Chekit-Q-fever, IDEXX, Liebefeld-Bern, Switzerland), following a protocol prescribed by the manufacturer. A sample with the sample-to-positive (S/P) ratio  $\geq 40$  was defined as positive. The Se and Sp estimates of this

diagnostic test were obtained from a previous study (Paul et al., 2013). This information was used to generate the corresponding Beta distribution from the BetaBuster software ([www.epi.ucdavis.edu/diagnostictests/betabuster.html](http://www.epi.ucdavis.edu/diagnostictests/betabuster.html)).

The following data was obtained from the Danish Cattle Database: Birth date, slaughter date, breed, gender, herd of origin (i.e. herd where the animal was born), delivering herd (i.e. herd from where the animal was sent for slaughter), type of delivering herd (i.e. dairy vs. beef herd), number of movements (i.e., how many times an animal was moved from one herd to another since birth to slaughter) and local cattle density measured, as the total number of animals and herds within circles of a 1 or 5 km radius of the herd of last housing before slaughter.

## Statistical analysis

### *Prevalence model*

The prevalence was estimated in a Bayesian analysis based on the model used by Okura et al. (2011). Because blood ELISA is not a perfect test (Paul et al., 2013), we accounted for the uncertainty of the diagnostic test in the models. The apparent antibody status of an animal was linked to the true (but latent) antibody status in terms of the Se and Sp of the diagnostic test. Thus, the test result for the  $i$ th animal of the  $j$ th breed is:

$$\text{result}_i \sim \text{Bernoulli}(AP_j)$$

$$AP_j = \text{Se} \times TP_j + (1 - \text{Sp}) \times (1 - TP_j)$$

$$\text{Se} \sim \text{Beta}(56.02, 11.48)$$

$$\text{Sp} \sim \text{Beta}(125.79, 2.26)$$

Two models were developed to estimate the  $TP_j$ : (1) a model assuming that prevalence varied among the breeds, i.e. a fixed effect model, where:

$$TP_j \sim \text{Beta}(1, 1)$$

and (2) a model assuming within breed prevalence (breed specific prevalence) was similar, i.e. a random effect model, where:

$$\text{logit}(TP_j) = \alpha_j$$

$$\alpha_j \sim N(\mu, \tau)$$

Non-informative priors for both mean  $\mu \sim \text{Norm}(0, 0.001)$ , and precision  $\tau \sim \text{Gamma}(0.01, 0.01)$  were modeled in the random effect model using the OpenBUGS (Thomas et al., 2006) parameterization. We also estimated AP and TP specific for gender, breed group and herd type from the fixed effect model. Prior Beta distributions for the Se (Beta (56.02, 11.48)) and Sp (Beta (125.79, 2.26)) used in all models were obtained from the BetaBuster software using the Se and Sp estimates stated in Paul et al. (2013).

We ran all prevalence models in OpenBUGS, which acquires a Monte Carlo (MC) sample from the posterior distribution by employing a Markov Chain Monte Carlo (MCMC) sampling algorithm. The MCMC models were run for 20,000 iterations with the first 10,000 discarded as burn-in and the posterior inferences were calculated from the remaining MC samples. To assess convergence of the MCMC chain we visually inspected the time-series plots of selected variables as well as the Gelman-Rubin diagnostic plots using three sample chains with different initial values (Brooks and Gelman, 1998). Median and 95% posterior credibility intervals (PCI, the Bayesian analogous of a confidence interval) of AP and TP were calculated to draw posterior inferences. The hypotheses of the differences between prevalences in different trait groups were tested by calculating the Bayesian posterior probabilities (POPR) e.g.,  $H_0: TP_{\text{female}} > TP_{\text{male}}$  or  $H_0: TP_{\text{dairy}} > TP_{\text{beef}}$ . The Bayesian PORP used to test the  $H_0$  was calculated using the Step function in OpenBUGS model and interpreted as the proportion of MC samples for which  $H_0$  was true. For example, if the PORP for  $H_0: TP_{\text{female}} > TP_{\text{male}}$  was 0.98, it can be interpreted as for 98% of the MC samples the  $TP_{\text{female}}$  was greater than  $TP_{\text{male}}$ . In other words, it can be interpreted as statistical significance in a one sided test.

### **Risk factor model**

The effect of possible risk factors extracted from the Danish Cattle Database on the probability of *C. burnetii* seropositivity was assessed by a Bayesian logistic regression model. We accounted for the uncertainty of the diagnostic test in this model as we did in the prevalence models. The true (but latent) antibody status of an animal was used as a dependent variable in the logistic regression model:

$$\text{result}_i \sim \text{Bernoulli}(AP_i)$$

$$AP_i = Se \times TP_i + (1 - Sp) \times (1 - TP_i)$$

$$\text{Logit}(TP_i) = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2$$

$$Se \sim \text{Beta}(56.02, 11.48)$$

$$Sp \sim \text{Beta}(125.79, 2.26)$$

where,  $\alpha_0$  represents intercept,  $X_1$  and  $X_2$  are the risk factors for the  $i$ 'th animal; and  $\alpha_1$  and  $\alpha_2$  represent regression coefficients for  $X_1$  and  $X_2$  respectively.

First, univariable logistic regression was done by entering each risk factor in the model separately to assess the association between an independent variable and animal antibody status (positive vs negative). A variable was assessed significant if the 95% PCI value of the odds ratio did not include 1. Correlations among the variables found significant in the univariable analysis were assessed and variables with the correlation coefficient  $\leq 0.5$  in pairwise test were included in the multivariable model (Dohoo et al., 2003). Both forward selection and backward elimination procedure were used in the multivariable logistic regression to identify the significant risk factors. In forward selection procedure the independent variables were added to the model one by one and were kept in the model if the 95% PCI of the alpha estimate did not include 1. Check for biologically meaningful interactions were also done. Furthermore, model selection was also done by



comparing the deviance information criteria (DIC) before and after adding a variable in the model. Model with smallest DIC was selected for further analysis and a decrease in DIC  $\geq 5$  was deemed a significant improvement in goodness of fit of the model (Osterstock et al., 2008). In a univariable analysis DIC value of a model was compared with the DIC of the null model.

The Bayesian logistic regression models were developed in OpenBUGS. Posterior inference of parameter estimates and assessment of model convergence was done following the same procedure as used for prevalence models. However, few models which did not converge perfectly in 20,000 iterations were run for more iterations (up to 50,000) to achieve satisfactory convergence.

**Table 1:** Distribution of *Coxiella burnetii* antibody positivity in blood samples from cattle according to breed, gender, herd type and herd location collected between August and October 2012 from Danish slaughterhouses following a random sampling procedure.

	No. of positive samples	Total no. samples	Proportion (%) test positive
<b>Overall</b>	<b>44</b>	<b>800</b>	<b>5.50</b>
<b>Breed group</b>			
Dairy breeds	23	258	8.91
<i>Holstein</i>	17	207	8.21
<i>Jersey</i>	3	25	12.0
<i>Danish Red Cattle</i>	3	26	11.54
Beef breeds	13	315	4.13
<i>Aberdeen Angus</i>	1	27	3.70
<i>Charolais</i>	0	21	0.00
<i>Hereford</i>	2	80	2.50
<i>Limousine</i>	4	84	4.76
<i>Simmental</i>	4	61	6.56
<i>Other<sup>a</sup></i>	2	42	4.76
Crossbreeds	8	227	3.52
<b>Gender</b>			
Female	28	307	9.12
Male	16	493	3.25
<b>Type of delivering herds</b>			
Beef herds	32	716	4.47
Dairy herds	12	84	14.29
<b>Herd location</b>			
Funen	4	110	3.64
South and West Jutland	30	401	7.48
North and East Jutland	7	177	3.95
Zealand and Bornholm	3	112	2.68

<sup>a</sup>Includes all beef breeds with less than 10 animals

## Results

The 800 cattle slaughtered at six abattoirs originated from 753 herds and included cattle of 15 beef breeds and three dairy breeds i.e., Danish Holstein, Danish Jersey and Danish Red cattle. Thirty-nine per cent of the samples originated from beef cattle, 32% from dairy cattle and 29% from non-specified crossbreeds. Table 1 shows the distribution of the samples stratified by breed, gender, herd type and location. Breeds with less than 10 animals were excluded from the prevalence analysis. Only five beef breeds including Angus, Charolais, Hereford, Limousine and Simmental comprised  $\geq 10$  animals. Hence, 758 blood samples from 9 breeds were used for prevalence estimation. The overall proportion of test seropositive samples was 5.5% with noticeable variations between breeds, breed groups, gender and herd types. Discrete numerical variables such as number of movements; number of herds within a 1km and 5km radius; and number of animals within 1km and 5km radius were used as continuous variables. The median age of the cattle included in this study was 1.39 years with a lower quartile (Q1) and upper quartile (Q3) of 1.11 years and 2.49, respectively. The median age of female cattle was higher than that of male cattle. Summary statistics of continuous variables are presented in Table 2.

**Table 2:** Descriptive analysis of continuous variables on age, animal movements, herd size and density as used in a study of 800 blood samples from cattle collected between August and October 2012, at Danish slaughterhouses following a random sampling procedure.

	Median	Lower quartile (Q1)	Upper quartile (Q3)
Age (year)	1.39	1.11	2.49
Female	3.17	1.90	5.59
Male	1.19	0.98	1.42
No. of movements <sup>a</sup>	2	1	3
Herd size of delivering herd	56	23	133
No. of herds within 1km radius <sup>b</sup>	2	1	4
No. of herds within 5km radius	53	41	65
No. of animals within 1km radius	57	11	279
No. of animals within 5km radius	3481	1763	5962

<sup>a</sup> How many times an animal since birth was moved from one herd to another and to slaughter.

<sup>b</sup> Radius was calculated from the final herd before the animal was sent to the slaughterhouse.

## Prevalence model

Table 3 shows the posterior median estimated with 95% PCI of breed-specific AP and TP estimates, using a fixed effect model and the TP from a random effect model. The combined AP of three dairy breeds (9.11%) obtained from a fixed effect model was significantly higher (POPR=1) than that of beef breeds (4.32%) (Table 4). However, the difference between the APs of beef breed groups and crossbred cattle was not significant.

Similar trends were also observed in the TP estimated of different breed groups from the fixed effect model (Table 4). Significant differences between the AP and TP estimates were also noticed in female versus male and dairy herd versus beef herds (Table 4).

**Table 3:** Estimates of apparent and true prevalence of *Coxiella burnetii* seropositivity in different cattle breeds from 758 randomly selected blood samples from Danish slaughterhouses collected between August and October 2012.

	Apparent prevalence			True prevalence			True prevalence with random effect of breed		
	Prev. <sup>a</sup>	95% PCI <sup>b</sup>		Prev.	95% PCI		Prev.	95% PCI	
Aberdeen Angus	6.26	1.54	18.92	6.38	0.45	22.79	1.74	0.00	8.52
Charolais	4.30	0.88	16.33	3.87	0.15	19.31	1.51	0.00	7.62
Hereford	3.60	1.19	8.93	2.98	0.19	9.84	1.43	0.00	6.84
Limousine	5.58	2.15	11.60	5.51	0.93	13.22	1.83	0.00	7.76
Simmental	7.49	2.72	15.85	7.99	1.62	18.90	2.22	0.00	9.19
Holstein	8.49	5.21	12.76	9.26	4.63	15.13	3.82	0.00	10.33
Jersey	13.94	4.35	30.13	16.04	3.79	37.10	2.75	0.00	14.33
Danish Red Cattle	13.54	4.41	28.68	15.58	3.88	35.28	2.71	0.00	13.93
Cross breed	3.85	1.94	6.83	3.35	0.52	7.43	1.47	0.00	6.01
Overall	5.61	4.15	7.45	5.61	2.81	8.42	2.05	0.00	7.15

<sup>a</sup> Prevalence (%)

<sup>b</sup> 95% posterior credibility interval

**Table 4:** Estimates of apparent and true prevalence of *Coxiella burnetii* seropositivity in different categories from 758 randomly selected blood samples from cattle sampled at slaughter in Denmark, collected between August and October 2012.

	Apparent prevalence			True prevalence		
	Prev. <sup>a</sup>	95% PCI <sup>b</sup>		Prev.	95% PCI	
Gender						
Female	9.10	6.16	12.80	9.40	5.08	14.64
Male	3.62	2.21	5.52	2.61	0.26	5.50
Breed group						
Beef	4.32	2.41	7.18	3.54	0.51	7.39
Crossbred	3.90	1.98	6.77	2.98	0.32	6.86
Dairy	9.11	6.07	12.97	9.45	5.01	14.84
Type of delivering herds						
Beef herds	4.54	3.16	6.30	3.66	0.62	6.51
Dairy herds	15.10	8.53	23.64	16.67	8.06	27.86

<sup>a</sup> Prevalence (%).

<sup>b</sup> 95% posterior credibility interval.

## Risk factor model

The DIC value of the null model was 348.3. Based on the 95% PCI of the alpha estimate and DIC value, six variables, i.e. age (DIC=342.4), gender (DIC=332.2), breed group (DIC=327), type of delivering herd (DIC=339.9), number of movements (DIC=331.3) and herd location (DIC=334.1), were found significant in the univariable logistic regression analyses and they were all included in the multivariable model. Both forward selection and backward elimination procedures revealed the same final model. The result of the final multivariable model is shown in Table 5 (DIC=303.3). In the final model, age, breed group and number of movements showed significant association with animal antibody status.

## Discussion

In this study, both apparent and true prevalence of *C. burnetii* antibody positivity for different breeds of cattle raised for meat production delivered for slaughter in Denmark were estimated using Bayesian analysis. Moreover, we also estimated the effect of several risk factors for *C. burnetii* seropositivity using a Bayesian logistic regression model adjusting for diagnostic misclassification. The first part of the discussion is about methods and results of the prevalence models, followed by a discussion about risk factors. Finally, we discussed the usefulness of the statistical methods and data quality of this study.

**Table 5:** Multivariable Bayesian logistic regression analysis to identify risk factors associated with *Coxiella burnetii* seropositivity estimated from 800 randomly selected blood samples from cattle sampled at slaughter in Denmark, collected between August and October 2012.

	Beta	OR <sup>a</sup>	95% PCI <sup>b</sup>	
Age (year) <sup>d</sup>	0.33	1.39	1.12	1.57
Breed group				
<i>Dairy breeds</i>	1.28	3.59	1.69	8.63
<i>Crossbreds</i>	-0.01	1.00	0.37	2.52
<i>Beef breeds</i>	Ref. <sup>c</sup>	Ref.		
No. of movements <sup>d,e</sup>	0.49	1.59.	1.32	1.90

<sup>a</sup> Odds ratio.

<sup>b</sup> 95% posterior credibility interval.

<sup>c</sup> Reference category.

<sup>d</sup> OR calculated per unit change in measurement.

<sup>e</sup> How many times an animal moved from birth to slaughter.

## Prevalence estimation

Estimation of AP is a straight forward way of indicating the proportion of diseased animals. It does not require information about the Se and Sp of the diagnostic test, and uncertainty of AP estimates are narrower than the corresponding TP estimates. However, AP estimates of two studies are only comparable when both studies are similar in terms of study design and they use the same diagnostic test. Furthermore, AP estimates may underrate the true prevalence, thus, may mislead the belief of decision makers about the true presence of a disease. Hence, TP estimates are the frequently presented outcome of recent prevalence studies, where the test Se and Sp, along with their uncertainties are known.

The conclusion about the fixed effect prevalence models is that both AP and TP were significantly higher in dairy breeds vs. beef breeds, females vs. males and dairy herds vs. beef herds. The breed was also identified as a risk factor for *C. burnetii* seropositivity with higher prevalence in dairy breeds than beef breeds. The herd management system may be the primary explanation for these differences in prevalence, although the study does not rule out genetic factors. Beef cattle were maintained for a shorter management cycle than dairy cattle. Many beef cattle are slaughtered at a young age, apparently with a low probability of infection. In Denmark, most of the beef herds are small and extensively managed, as opposed to dairy herds. Moreover, beef cattle often spend a significant part of the year outside the farm buildings. This provides potentially more space for the beef cattle, and therefore, the exposure load probably remains low and may result in fewer infections. The small herd size could also be a reason for the lower seroprevalence in beef cattle; as *C. burnetii* seroprevalence was reported to increase with increasing herd size in previous studies (McCaughey et al., 2010; Paul et al., 2012; Ryan et al., 2011). However, we did not find herd size to be a risk factor in this study.

In this study, the TP estimates for dairy and beef breeds were 9.1% and 4.3%, respectively. These estimates could be compared to the prevalence estimates found in the other studies using serum ELISA. Very few studies have focused on *C. burnetii* seroprevalence in beef cattle in Europe (Alvarez et al., 2012; Kampen et al., 2012; McCaughey et al., 2010; Ruiz-Fons et al., 2010; Ryan et al., 2011). However, none of these were based on slaughtered cattle and did not calculate TP accounting for uncertainty of the diagnostic tests. The reported AP estimates in beef cattle from the studies in Europe varied from 0 to 18.3%, and some of the estimates were close to our findings (McCaughey et al., 2010; Ruiz-Fons et al., 2010). A study in Norway did not detect any cases of *C. burnetii* seropositivity in dairy and beef cattle (Kampen et al., 2012), while seroprevalence was 16.8% in beef cattle in Queensland, Australia (Cooper et al., 2011). APs and TPs within the dairy breed were similar, whereas these estimates came out to be much lower in beef breeds, as pointed out by the significant difference of prevalence between these two breed groups. It was expected that prevalence would be high in dairy breeds or cattle raised within the dairy herds, as previous studies in Denmark reported high prevalence in dairy herds and dairy cows (Agger et al., 2010; Paul et al., 2012). Indeed, 32% of cattle included in this study were dairy breeds, and 10% of the sampled cattle were raised in dairy herds.

For most of the breeds, the simulated TP estimates were close to the AP. The possible explanation for this is that we used prior values for the Se and Sp from a test with a reasonably high sensitivity (84%) and an almost perfect specificity (99%). But, the uncertainty around the TP estimates showed larger variation compared to AP estimates, as illustrated in Table 3. This variation is partly due to small sample size in some breeds, as also found in other studies (Okura et al., 2011; van Schaik et al., 2003). So, more data is required to get TP estimates with high precision. However, even in the larger breeds, uncertainties of TP estimates were larger than for AP estimates. Therefore, this variation might also be due to the uncertainty of the priors used for the Se and Sp, although we used a highly sensitive and specific test. To extract more information from the existing data, we imposed some restriction on the data as suggested by Okura et al. (2011), i.e. we developed a random effect prevalence model assuming that breed-specific prevalence was similar. The output of this model was in accordance with the results of the aforementioned study, i.e. estimates were moved towards the population mean prevalence, uncertainty of TP estimates became narrower, and as expected, the effect of the random effect of breed was more evident in breeds with fewer animals in the study population. However, the underlying assumption of within breed prevalence similarity might be questionable, as all breeds are not genetically identical and management systems vary according to the production type. Moreover, comparison of DIC values identified the model without random effect as a better fit than the random effect model.

## Risk factors

The age and number of animal movements were identified as risk factors of *C. burnetii* seropositivity in the final logistic regression model, along with breed groups. The model showed that the risk of positivity increased with increasing age, as also found in a previous study (Alvarez et al., 2012). A likely explanation is that the probability of exposure to the bacterium increases with animal age. Similar age effects were also observed in dairy cattle (Muskens et al., 2012; Paul et al., 2012). In contrast to other studies, where beef cattle were included as a subpopulation, age was not identified as a risk factor (Ruiz-Fons et al., 2010; Ryan et al., 2011). This variation may not only be due to differences in study designs in terms of population and sample sizes, but also different modeling approaches used. Both univariable and multivariable analyses confirmed our hypothesis that the risk of seropositivity increased with an increasing number of movements. A probable explanation is that it is not the transport per se, but that the accumulation of animals from different herds increased the risk of exposure, simply because some animals may excrete the bacterium.

In recent years, the Bayesian approach has been used frequently to account for test uncertainty in estimation of TP, and for risk factor studies (Enøe et al., 2000; Tu et al., 1999). The Bayesian models of this study were flexible and allowed us to estimate TP and associated risk factors from fixed and random effect models. The ELISA used in this study has a high Se and Sp when the target condition is detection of *C. burnetii* seropositive animals (Paul et al., 2013). The Se and Sp of this test were achieved from a diagnostic test evaluation study using a Bayesian framework in the Danish cattle population. With the inclusion of risk factors in the logistic regression model, the posterior estimates of the Se (83%) and Sp (98%) did not change significantly. This indicates that the test characteristics are less likely to be influenced by these covariates. Descriptive analysis

showed that all regions of Denmark were well represented by the samples used in this study. The animal and herd information are recorded and routinely updated in the Danish Cattle Database, and farmers are obliged to report this information immediately after the birth of an animal.

## Conclusion

The prevalence of *C. burnetii* seropositivity in cattle raised for meat production and sent to slaughter houses in Denmark was lower than the prevalence in Danish dairy cows. The prevalence in slaughtered cattle of dairy breeds was within the range of what was found in Danish dairy herds, while beef breeds showed a much lower prevalence. Therefore, for exploring the further epidemiological information, future research should be focused more on dairy cattle than on beef cattle. Age and animal movement were also found to have significant associations with the level of *C. burnetii* antibodies. Similar AP and TP estimates for most breeds explained that estimation of AP is a reliable measure when a highly sensitive and specific diagnostic test is used. Hence, results from the seroepidemiological studies of *C. burnetii* in cattle carried out using the same ELISA as in the present study may not need to be adjusted for diagnostic test uncertainty.

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## **CHAPTER 7**

### **GENERAL DISCUSSION**

#### **SYNOPSIS**

This chapter summarises the knowledge derived from the previous chapters on research and literature review and discusses the perspectives for further research on the epidemiology of *Coxiella burnetii* infection, and options for practical applications in surveillance and control of the infection.



## Introduction

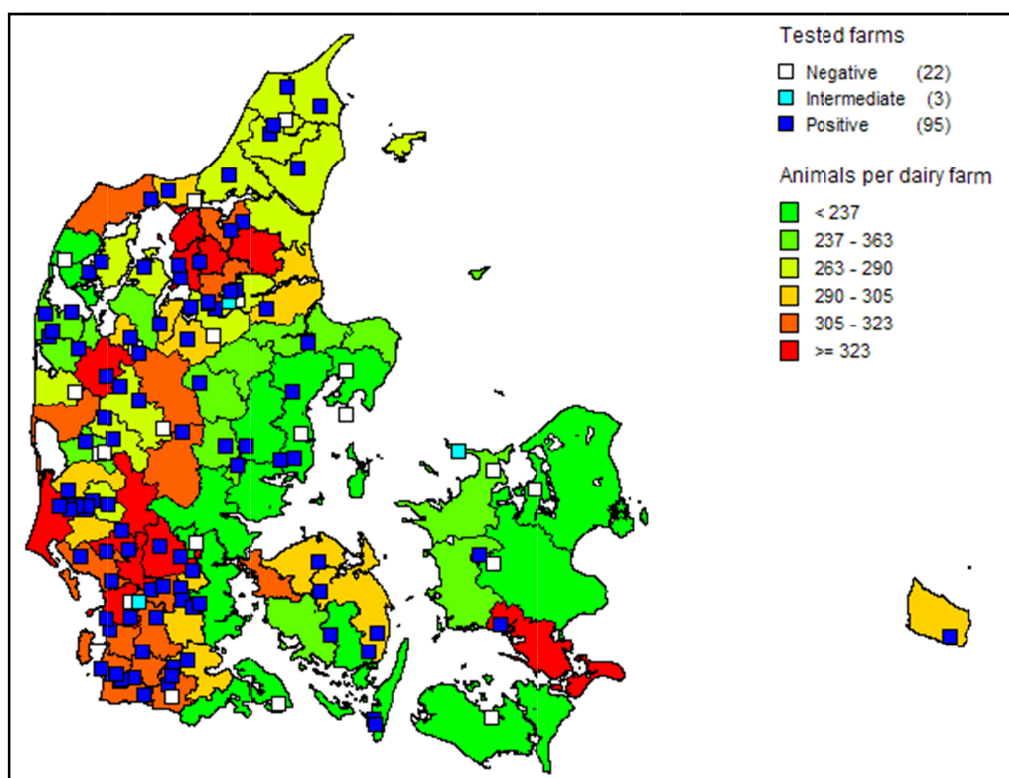
Emerging infectious diseases (EID) with the ability to produce a serious epidemic are highly prevalent worldwide. More than 60 percent of EIDs, including Q fever are zoonotic and have the ability to exhibit negative social and economic impacts (Jones et al., 2008). It is being regarded as a formidable challenge to prevent EIDs in a new environment, but possible control measures can be installed to mitigate their impact on animals and humans. Epidemics may last for a few weeks or a few months and can overwhelm the existing situation. In the early stage, the mechanisms of the process of disease emergence and consecutive diffusion remain nearly unknown, and could vary over time (Gonzalez et al., 2006). A multidisciplinary reaction across sectors, professions and functional roles is the key element for planning how to manage the disruptions and disasters caused by an EID. Targeted surveillance, disease management and an adoptive control program for EIDs requires adequate information (data) on emergence, spread and the persistence of diseases (Bonneux and Damme, 2011). A multidisciplinary approach, when supported by evidence of the observed virulence, epidemiological indices and effectiveness of the control measures assists in reducing the lag time between detection of pathogens, laboratory verification and response (Gonzalez et al., 2006). Complete field based observational studies are essential for obtaining knowledge of emergence, spread and persistence of a disease. Questions related to the mechanisms behind the process of emergence, spread and persistence of the disease cannot be answered sufficiently by the information gained from the observational studies, which are incomplete or of an exemplary situation (Gonzalez et al., 2006).

Q fever is endemic in many European countries (ECDC, 2010; Frankel et al., 2011) and is considered as an emerging or re-emerging disease in countries such as the Netherlands and France (Frankel et al., 2011; Roest et al., 2011). In the Netherlands, the emergence of Q fever was notified in humans in 2007 with a peak occurrence in 2009, despite the fact that the disease was considered endemic for several decades (Roest et al., 2011). However, adoptive veterinary control measures based on previous knowledge were able to reduce the magnitude of the epidemic in the subsequent year (Roest et al., 2011). Since the recent epidemic in the Netherlands, epidemiological research on Q fever in both animals and humans has gained sufficient attention from researchers in many European countries, including Denmark. In Denmark, like in most other European countries, the existence of *C. burnetii* has been reported for several years. However, information on virulence, and epidemiological indices such as emergence, prevalence, spread and persistence of infection in different hosts based on field observations were limited. Thus, field based epidemiological research on Q fever was necessary to assess the occurrence and to explore the risk factors of the disease in Denmark. Acquired knowledge from the studies included in this thesis may support developing targeted surveillance, disease management and an adoptive control program in the future.

The overall objective of this thesis was to investigate the epidemiology of *C. burnetii* seropositivity in Danish cattle with a focus on the occurrence and the risk factors in a series of field based observational studies.

## Data quality and statistical tools

The study designs and the sample size calculations of the studies included in this thesis were based on the purpose of the respective studies. Cross-sectional designs were used to calculate prevalence in dairy herds (Chapter 3) and in slaughtered cattle (Chapter 6). To ensure the representativeness of the target population and to avoid selection bias, study units were selected randomly in both of these studies. A relatively high allowable error ( $l=0.10$ ) was used to estimate the sample size for calculating herd level prevalence. The choice of such high allowable error was in accordance with the economics of the project. It reduced the sample size; however, it also induced low precision of the parameter estimates. For calculating sample size in chapter 6, allowable error  $l=0.05$  was used. All major administrative divisions of Denmark were covered by the random sample (Figure 1).



**Figure 1:** Distribution of 120 randomly selected Danish dairy herds tested for antibodies against *Coxiella burnetii* in July 2012, and a regional number of cattle per km<sup>2</sup> (all cattle types).

Chapter 4a identified several herd level risk factors associated with *C. burnetii* antibody positivity in BTM. However, this study was based on the cross-sectional design, and therefore, could not determine the causality with the same strength as had it been a cohort study (Ersbøll et al., 2004). The 100 herds included in this study resulted in wide confidence intervals for parameter estimates of management and biosecurity factors found to be significant by logistic regression analysis. Results indicate that precision of the parameter estimates could be improved by increasing the sample size. Another herd risk factor analysis was also included in Chapter 3, although the main focus of this chapter was to estimate the dairy herd prevalence in 2012, and to compare it with the prevalence found in a previous study in 2008 (Agger et al. 2008). However, this risk factor analysis included

only those variables readily available in the Danish Cattle Database, aiming particularly to identify an association between cattle density and *C. burnetii* antibody positivity in BTM. Whereas, the risk factor analysis in Chapter 4a mainly focused on the relationship between management and biosecurity factors and *C. burnetii* antibody positivity in BTM. Only variable 'Herd size' was common in these two risk factor analyses. Hence, the overlap between the datasets of these two studies was minimal. Therefore, the outcome of these two studies can be considered to be complimentary to each other. A cross-sectional study with a follow-up was implemented to assess the factors associated with seropositivity in dairy cows, and to estimate the within herd seroprevalence in 24 dairy herds (Chapter 4b). The included herds were selected by a stratified random sampling procedure from three strata, based on the level of antibodies in BTM samples (BTM antibody positive, negative and intermediate). Along with this, the inclusion of all lactating cows from the selected herds might probably minimise the selection bias, if any. But, due to the inclusion of only 24 herds, uncertainty around the parameter estimates of herd level variables in the statistical analysis was very high. Moreover, although selected randomly, the number of herds selected from each stratum was not proportional to the proportion of the corresponding stratum in the initial population (Paul et al., 2012); and the BTM statuses of the herds were not followed repeatedly. Hence, the overall seroprevalence of within herd seropositivity could not be estimated. The rationale behind the inclusion of herd level risk factors was that the cows within a herd share common management traits. The management routine might differ from herd to herd, and might influence the risk of infection of individual cows. For some of the included cow level factors such as fat and protein contents and individual milk yield, it was difficult to establish causal associations with *C. burnetii* antibody status in the individual cow. Individual milk yield was included in the analysis to see its dilution effects on antibody concentration in the milk samples. Inclusion of fat and protein contents in the analyses was due to the use of milk yield without adjustment for energy. The variable 'Leg problems' was used as a proxy for the farmers' attitudes towards farm management and hygiene. *C. burnetii* is reported to be able to cause mastitis. It was hypothesised that this organism also has association with other udder problems and hence, 'udder problems' were included in the analyses. The management of cattle raised for meat production in Denmark is different from dairy cattle production. The risk factors for *C. burnetii* infection in Danish slaughter cattle might be different from that for Dairy cattle. Thus, to evaluate risk factors for *C. burnetii* in Danish slaughter cattle, an analysis was also carried out in Chapter 6. This study also included only those variables readily available in the Danish Cattle Database.

The diagnostic test evaluation study (Chapter 5) also followed a cross-sectional design involving 17 dairy herds. The selection procedure for herd selection was described in Chapter 4b. The three strata (BTM positive, BTM negative and BTM intermediate herds) were used as subpopulations with an assumption of differences in animal-level prevalence. The selected study units and study population, therefore, fulfilled the criteria to be involved in this study.

Supplementary registered data from the Danish Cattle Database was used in all of the studies included in this thesis. Registered data was already collected, and hence, it was cheap and available. Detailed information regarding farm management practice, however, is not routinely collected. Therefore, information on herd management practice was necessary to answer the objectives set in Chapter 4a and Chapter 4b. Thus, information on

herd level management was gathered from the farmers in structured telephone interviews with questions at herd level. Data obtained from the questionnaire interviews might have introduced recall bias, due to farmers' loss of memory which could not be ruled out.

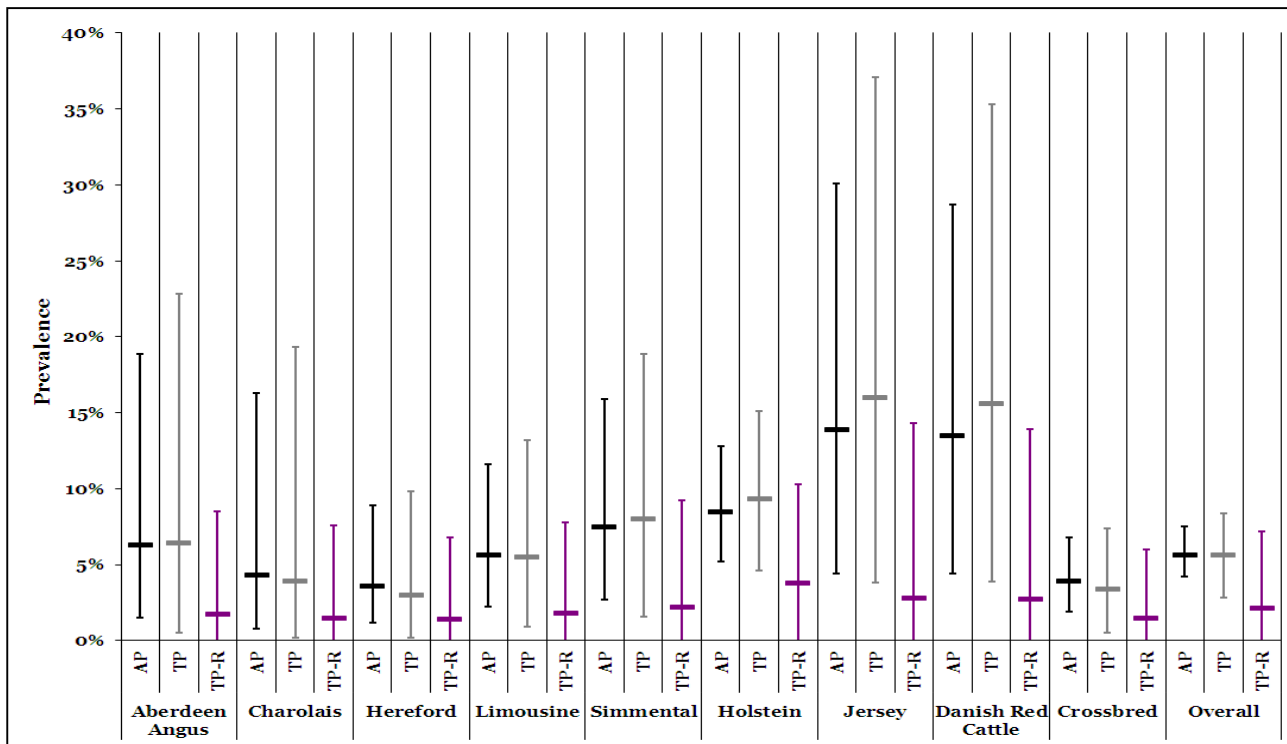
Apparent herd prevalence (Chapter 3) and within herd prevalence (Chapter 4b) was estimated as the proportion of test positive animals among the total number of animals tested, assuming 100% sensitivity (Se) and specificity (Sp) of the diagnostic test. The true prevalence (apparent prevalence adjusted for the diagnostic test uncertainty) could not be calculated due to the lack of reliable Se and Sp estimates during the study periods, which might introduce bias in the parameter estimates (Greiner and Gardner, 2000; Tu et al., 1999). A Bayesian approach of prevalence estimation is a widely used statistical tool in this context where misclassification errors due to a suboptimal diagnostic test can be taken into account (Enøe et al., 2000; Speybroeck et al., 2012). This approach was used to estimate the true prevalence of seropositive animals in Danish slaughter cattle (Chapter 6), where estimates were adjusted for test uncertainty evaluations in a previous study (Paul et al., 2013). Logistic regression analyses were used to identify the factors associated with *C. burnetii* antibody positivity in dairy herds (Chapter 4a) and cows (Chapter 4b). Logistic regression is a traditional and widely used statistical tool for identifying risk factors for diseases (Dubuc et al., 2010; Saa et al., 2012; Van den Brom et al., 2013). However, a potential problem is that misclassification of the study units may take place due to lack of perfection of the diagnostic test (Greiner and Gardner, 2000). Hence, it is likely that some herds or cows have been included as positive to *C. burnetii* antibodies in the studies when in fact they were not positive (false positive), and vice-versa (false negative). This might bias the results, and it is likely that the effect has been diluted and that the estimated effects of risk factors on *C. burnetii* positivity were conservative. It is recommended that the Se and Sp estimates of diagnostic tests, if known, should be taken into account in logistic regression models to obtain the true effect of risk factors (McInturff et al., 2004). This was implemented in assessing risk factors of *C. burnetii* seropositivity in Danish slaughter cattle (Chapter 6). The performance of the diagnostic tests used in different studies of this thesis was evaluated using the Bayesian approach of latent class analysis. This is a recommended and commonly used approach of diagnostic test evaluation, which can minimise biases in the parameter estimates and does not require a gold standard (reference) test (Enøe et al., 2000).

## **Synthesis of epidemiological findings of *Coxiella burnetii* antibody positivity in Danish cattle**

### **Magnitude in cattle**

The apparent prevalence of *C. burnetii* antibody positive Danish dairy herds was 79% in 2012 (Chapter 3). This prevalence was significantly higher than the prevalence (59%) found in 2008 (Agger et al., 2010). The latter study concluded an increasing trend in the seroprevalence or prevalence of antibody positivity from 2003 through 2008, by comparing its results with the results of other studies based on clinically suspected samples. This conclusion is supported by the results of the 2012 study (Chapter 3). Reporting of only clinical cases of Q fever in cattle was made mandatory in Denmark in 2005. However, as there were no clear indications of serious zoonotic potential and

economic impact, no further national or regional surveillance or control programs (beyond notification of clinical cases) have been implemented. The estimated herd prevalence in 2012 in Denmark was within the range of prevalence reported from different European countries in recent years (EFSA, 2012; Guatteo et al., 2011), but was one of the highest estimates, if not the highest, among them. Herd positivity was found in all major administrative regions of Denmark, and no regional differences in prevalence were identified (Figure 1). Herd density was not associated with the herd test categories. These two findings were consistent with the findings of the 2008 study (Agger et al., 2010). Findings of these two studies might indicate homogeneous distribution of *C. burnetii* in Denmark and might probably indicate no spatial clustering. However, cluster analysis was not included in any of these two studies. It was observed that prevalence increased with the increasing average herd size of dairy herds. Herd size has been identified as a risk factor for many diseases, including Q fever. The effect of herd size on *C. burnetii* infection in Danish cattle is discussed later in this chapter.



**Figure 2:** The posterior median and 95% posterior credibility interval for the apparent prevalence (AP), true prevalence (TP) and true prevalence using a random effect model (TP-R) for different breeds estimated from 758 blood samples of slaughtered Danish cattle collected between August and October 2012.

Stratum specific (low, intermediate and high BTM antibody levels) within herd seroprevalence showed a high prevalence in initially BTM positive (27%) and initially BTM intermediate (23%) herds; and a very low prevalence in initially BTM negative herds (0.9%) (Chapter 4b). Within herd prevalence, in initially BTM positive and BTM intermediate herds did not change significantly over time, i.e. for an 11-month follow-up period (Chapter 4b); which might indicate long-lasting stability of within herd seroprevalence, if the infection is introduced to a population. A significant increase in



within herd prevalence was observed in herds which were initially BTM negative. A careful evaluation of the data indicated that these abrupt increments of within herd prevalence were observed in only two out of ten negative herds. Newly purchased cattle were not quarantined in one of these two herds; whereas, the other herd did not purchase new animals during a one-year period before data collection. Therefore, it was not conclusive for these two herds that purchasing new animals or lack of quarantine contributed to this sudden increase of within herd prevalence. Nevertheless, these changes in within herd prevalence indicated different dynamics in some of the cows in these herds, i.e. conversion of their antibody status during the study period.

The overall true seroprevalence in Danish slaughter cattle was 5.6%, regardless of breed, type of delivering herd and gender (Chapter 6). A significant difference of prevalence was observed between the slaughtered dairy cattle (9.45%) and of beef (3.54%) breeds. Prevalence in all major dairy breeds (e.g., Danish Holstein, Jersey, and Danish Red Cattle) was higher than the population average (Figure 2). The overall findings of a higher prevalence of the *C. burnetii* antibody positivity in dairy breeds agree with the findings from other seroprevalence studies (McCaughey et al., 2010; Ryan et al., 2011). Genetic differences among the cattle breeds might be a possible explanation for these variations between dairy and beef types of cattle. Management factors might also contribute to this breed variation, as management of dairy and beef cattle productions are different in Denmark. This was also reflected by the finding of a difference in prevalence between types of delivering herds, i.e. higher prevalence in dairy herds (16.67%) than in beef herds (3.66%). However, a study in Norway did not find any prevalence either in dairy or in beef cattle (Kampen et al., 2012). The effect that breed has on *C. burnetii* seropositivity in Danish cattle has been discussed further in the risk factor section of this chapter, since this variable was also included in the risk factor analyses of individual cow level study (Chapter 4b) and Danish slaughter cattle study (Chapter 6). It was also found that female cattle (9.40%) contributed more in overall prevalence than males (2.61%). The difference of prevalence between female and male slaughter cattle might be due to the fact that females were relatively older (median age 3.17 years) than males (median age 1.19 years). Therefore, it was hypothesised that the seropositivity of *C. burnetii* could be better explained by the age effect rather than the gender effect. McCaughey et al. (2010) also did not find any gender related effect on the *C. burnetii* seroprevalence in cattle.

### **Risk factors of *C. burnetii* antibody positivity in dairy cattle herds**

Herd factors found to be associated with the level of *C. burnetii* antibodies in the BTM of Danish dairy herds were: 1) use of common farm equipment, 2) contact with visitors (with people from outside the farm), 3) insemination by people other than an AI technician, 4) herd health contract with the veterinarian, and 5) hygienic precautions (changing boots/clothes etc.) by the veterinarian before entering the barn. Increased antibody positivity was observed in herds where animals came in contact with visitors, where farm utensils were shared with other herds and in herds with a herd health contract for routine health checks by the veterinarian. The hypothesis is that visitors, herd veterinarian and common equipment might mechanically transmit *C. burnetii* bacteria into the herd they enter. A review article stated the possibility of mechanical transmission of bacteria (Woldehiwet, 2004). However, contrary to this, Taurel et al. (2011) did not find any association between herd antibody status and animals' contact with farm personnel and sharing common equipment. The AI technicians probably have a high level of

working-hygiene, and hence, the probability of transmission of the agent to herds where cows were inseminated by the AI technicians was low. It was also found that the probability of a herd being positive declined when improved hygienic precautions were taken by the herd veterinarian before entering the barn. A similar finding was also reported in a cow level study in Denmark (Paul et al., 2012).

Additional to the above mentioned factors, herd size, stable type, number of workers, use of maternity and disease pens, purchasing animals and quarantine practice were also found to have significant associations with herd antibody status in univariable analyses. In some previous studies, increasing herd size (McCaughey et al., 2010; Paul et al., 2012; Ryan et al., 2011) and loose housing (Czaplicki et al., 2012) were shown to have a positive relationship with antibody positivity in herds. Contrary to the findings of the current study, 'purchasing new animals' and 'quarantine of newly purchased animals', were not found significant in a study by Taurel et al. (2011). Random movement of the animals in loose housing probably results in closer contact between non-infected and infected animals and contaminated environments than in tie stalls. Thus, transmission probability is relatively high in a loose housing system. Maternity pens might be used for diseased animals - and they are possibly not being cleaned and disinfected properly, hence, the transmission probabilities increase. The purchase of animals (introducing new animals) is a classical factor for introducing a new infection into herds, as reported in Nielsen et al. (2007). However, this can be prevented if the newly purchased animals are properly quarantined.

### **Factors associated with *C. burnetii* antibody positivity in cattle**

Several factors that were unravelled were closely associated with *C. burnetii* seropositivity in Danish dairy cows (Chapter 4b), and cattle slaughtered for meat production (Chapter 6). Among those, some factors were individual animal traits and some were herd level factors (management factors). Cow-level factors that found to have significant association were breed, parity, milk yield, fat and protein content in milk. Seropositivity in slaughtered cattle was positively associated with age, breed group and number of movements. Among the herd-level factors, herd size, stable type, quarantine of newly purchased animals and improved hygienic precautions taken by the veterinarian before entering the barn (washing hands and changing clothes and boots before entering the barn) were associated with positivity in dairy cows. In addition, the season was significantly associated with *C. burnetii* antibody positivity in Danish dairy cows. One of the important features of epidemiology of *C. burnetii* antibody positivity in Danish dairy cows is that somatic cell count and reproductive problems were not found to be associated with an increased risk of antibody positivity in cows. Identified risk factors to a great extent demonstrated similarity with factors unravelled in other countries, but some differences were also observed. These country specific differences probably indicate a different epidemiological profile of *C. burnetii* antibody positivity in Danish dairy cows, and the underlying mechanisms explaining its distribution and dissemination are yet to be defined.

Danish Holstein cows have a higher risk of being *C. burnetii* antibody positive than Jersey cows, and these results correspond with the findings of a study in Northern Ireland

(McCaughey et al., 2010). Significant variations in breed susceptibility were also seen in the Danish slaughtered cattle, with a higher prevalence in dairy breeds than beef breeds. Genetic variation among the breeds is a probable reason for this breed variation. However, further research is required to confirm this hypothesis. Variation of seroprevalence between dairy and beef breeds may also be influenced by the herd management system. Danish beef cattle are generally managed extensively in relatively smaller herds as opposed to dairy herds. Beef cattle often spend a significant part of the year outside the farm buildings, which gives them potentially more space and minimises the buildup of a sufficient exposure level of bacterial density. The small herd size could also be a reason for the lower seroprevalence in beef cattle, as seroprevalence was reported to increase with increasing herd size in previous studies (McCaughey et al., 2010; Paul et al., 2012a; Ryan et al., 2011).

*C. burnetii* seroprevalence increases with increasing age in both dairy cows and cattle slaughtered for meat production. The probability of being exposed to the bacterium increases with the age of the animal. The longer the animal lives, the higher the chances of being infected or having seroconverted. So it is likely that prevalence will be higher in older cattle than younger. A positive age relation with *C. burnetii* seropositivity has also been found in other studies (Alvarez et al., 2012; Bottcher et al., 2011; Capuano et al., 2004; Guatteo et al., 2007; Muskens et al., 2012). In contrast to these, Ruiz-Fons et al. (2010) and Ryan et al. (2011) did not find age as an important factor for seroprevalence.

It was observed that the seroprevalence increased with an increasing number of movements before the cattle were slaughtered. It might not be the transport *per se*, but it is probably due to the accumulation of cattle from different herds that increased the risk of exposure, simply because some animals may excrete the bacterium.

A negative association between milk yield and seroprevalence was identified in dairy cows contrary to the findings of Garcia-Ispuerto et al. (2011). Dilution factors on antibody concentration in milk samples, rather than biological factors, might be a possible explanation for this association. Antibodies are likely to be diluted more in high yielding cows than in low yielding cows, and hence, the chance of being identified as positive reduces in high yielding cows, even if they are positive.

Seroprevalence increased with increasing protein and decreasing fat content in raw milk in dairy cows. However, the exact mechanisms for such findings are not yet known. Thus, further investigation is necessary to clarify the reasons behind these associations. Contrary to this finding, Rose et al. (1994) found a negative association between milk protein content and the level of milk antibodies.

A temporal trend in the occurrence of *C. burnetii* antibody positivity was observed in Danish dairy cows, with a higher risk in the summer (Chapter 4b). Occurrence declined steadily to the lowest level in winter. Results indicated that the occurrence of *C. burnetii* seropositivity in Danish dairy cattle followed a consistent decrease in temperature, distinguishing the transition from summer to winter. High ambient temperatures in summer might probably favour the growth of the bacteria. Moreover, high ambient temperatures might also have an influence on tick burden, which passively influences the

occurrence of *C. burnetii* infection. Ticks can transmit *C. burnetii* mechanically, and the organism has been isolated from more than 40 species of ticks (Angelakis and Raoult, 2010; Lang 1990; Maurin and Raoult, 1999). However, the effect of a tick burden on the occurrence of *C. burnetii* seropositivity was not looked at in this study. Seasonality in the occurrence of *C. burnetii* infection has been reported in cattle in Cyprus and Japan (Cantas et al., 2011; Yanase et al., 1997). The temporal trend found in Cyprus was as similar as the present study, whereas, an opposite trend, i.e. highest occurrence in winter and lowest in summer, was found in Japan. Temporality was also described in recent outbreaks of Q fever in small ruminants and humans in the Netherlands. However, these seasonal outbreaks were said to be associated with lambing or kidding seasons (Roset et al. 2011); where in Denmark, there is no specific calving seasons. Consequently, an identified temporal trend in the occurrence of *C. burnetii* seropositivity in Denmark might not be associated with seasonal calving.

Among the herd-level factors, i) increasing herd size, ii) cows raised in loose housing systems, iii) cows from herds where quarantine was not practised for newly purchased animals and iv) cows from herds where veterinarians did not take adequate hygienic precautions (washing hands and changing clothes and boots before entering the barn), were positively associated with *C. burnetii* antibody positivity in dairy cows. Identified risk factors indicate that factors associated with herd management and biosecurity can act as contributors for *C. burnetii* infection in Danish dairy cattle.

The positive association of herd size with *C. burnetii* infection in cattle was also reported by McCaughey et al. (2010) and Ryan et al. (2011). Increasing the number of cows in a herd might increase the transmission probability between cows in close proximity. However, herd size was not identified as an influencing factor of *C. burnetii* seropositivity in Danish cattle used for meat production.

It was observed that cows housed in loose housing systems were at higher risk of being *C. burnetii* seropositive than cows housed in tie stalls. In a loose housing system, there is more space than in a tie stall housing system, and therefore, the probability of random contact between uninfected and infected animals and between uninfected animals and contaminated environments is also higher. Hence, the risk of transmission is probably increased in loose housing systems. A similar finding was also reported in a study in Belgium (Czaplicki et al., 2012).

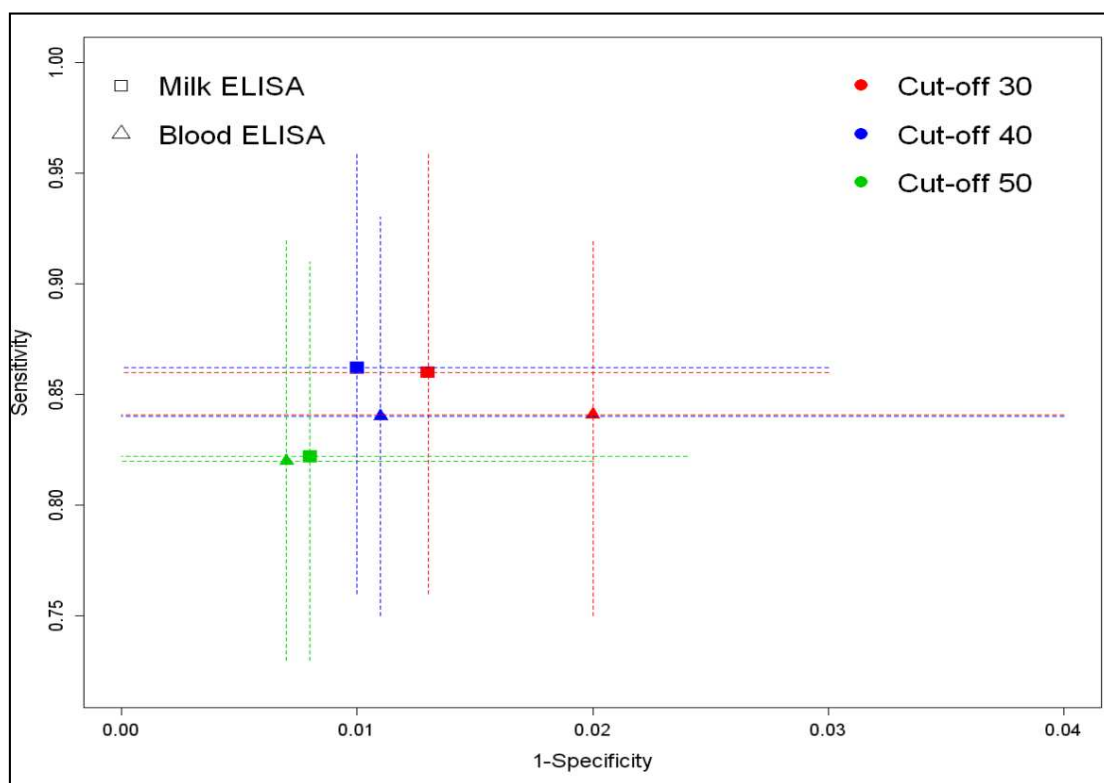
It was also found that quarantine of newly purchased cattle reduced the occurrence of *C. burnetii* seropositive in Danish dairy cows. The introduction of new animals in a herd increases the risk of introducing *C. burnetii* infection into cattle herds (EFSA, 2010). Therefore, practising quarantine for purchased animals is an important step to prevent the introduction of infection in a herd.

A review article by Woldehiwet (2004) stated that herd veterinarians, as a mechanical carrier, might transfer the pathogen from infected to non-infected herds through, e.g. boots, clothing, etc. Therefore, Improvement of hygienic precautions by a herd veterinarian is an important biosecurity measure to prevent or to reduce the chance of introducing a new infection into a herd. This was also reflected in the current study

(Chapter 4b), where it had been shown that a deficit in hygienic precautions, such as not changing clothes or boots increased the chance of a cow being *C. burnetii* seropositive.

### Evaluation of the performance of diagnostic tests

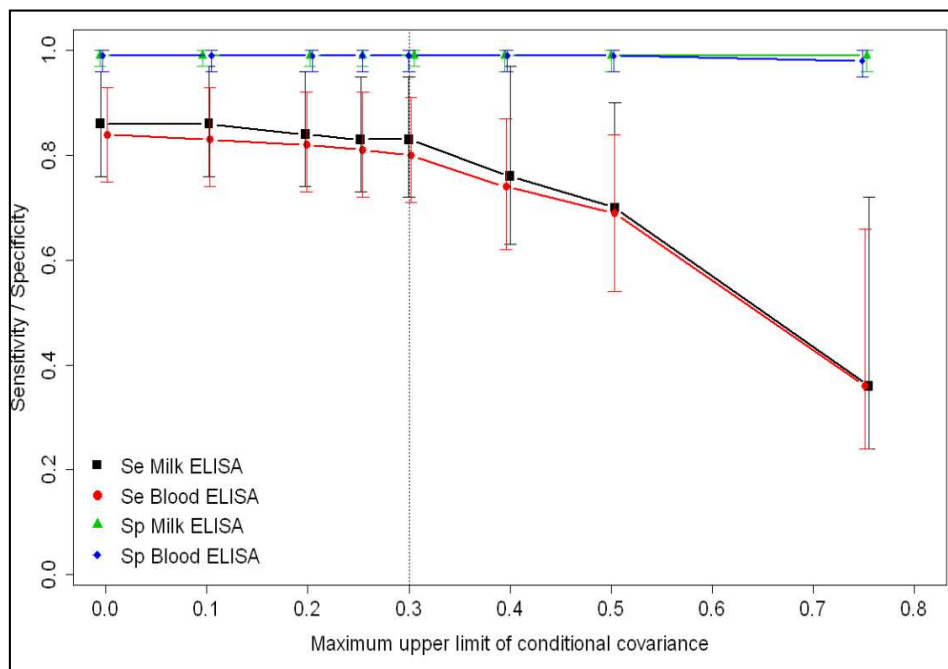
The performance of a commercially available ELISA, the diagnostic test kit used in all the studies in this thesis, was evaluated by latent class analysis using a Bayesian framework. Results showed that both milk and blood ELISAs are equally highly sensitive and specific in all the cut-offs used. Comparison of the test parameter estimates obtained by using three different cut-offs (i.e.,  $S/P \geq 30$ ,  $S/P \geq 40$ ,  $S/P \geq 50$ ) showed that the best combination of the Se and Sp was obtained at cut-off  $S/P \geq 40$  for both tests. At this cut-off, the Se and Sp of milk ELISA was 0.86 and 0.99, respectively; whereas the Se and Sp of blood ELISA was 0.84 and 0.99, respectively. The difference between the Se estimates of these two tests was not statistically significant. The Se and the Sp estimates did not change when cut-off  $S/P \geq 30$  was used, but the Se reduced slightly at cut-off  $S/P \geq 50$  (Figure 3). Differential positive rate (DPR) estimates also indicated that cut-off  $S/P \geq 40$  should be used to obtain the best test performance for classifying the serological status of a cow.



**Figure 3:** Sensitivity and 1-Specificity plots of milk and blood ELISAs using sensitivity and specificity estimates from conditionally independent models at different cut-offs.

All of these estimates were obtained from a model which assumed that these two tests are conditionally independent (CID). This assumption may not be valid, as both tests were detecting the same biological element (antibodies) in two different media (milk and blood). A different conditionally dependent (COC) model was therefore developed to

evaluate this assumption. Analyses showed that the Se and Sp estimates of the tests did not change unless a very high proportion of conditional covariance ( $\geq 40$ ) was used (Figure 4). This could be explained by the fact that these two tests may be biologically dependent; however, this is not an absolute dependence due to the high Se and Sp of both tests. Thus, this correlation does not affect the parameter estimates when tests are evaluated against each other. Therefore, estimates obtained from the CID models at cut-off S/P  $\geq 40$  seem valid.



**Figure 4:** Plots of the posterior mean estimates of the sensitivity (Se) and specificity (Sp) of blood and milk ELISAs from conditionally dependent (COC) models with varying proportions of the maximum conditional covariance.

## Practical application of the findings

The situation of *C. burnetii* seropositivity (an indirect indication of infection) should be addressed more in Denmark than it is presently. Reporting *C. burnetii* infection in cattle is mandatory in Denmark (Anonymous, 2010). Epidemiological investigations conducted on *C. burnetii* seropositivity in Denmark in 2008 and 2012 revealed a very high herd level prevalence and identified an increasing trend of herd level seroprevalence in dairy herds. Thus, the country should not rely only on reported clinically suspected samples. In order to achieve a complete scenario, it is imperative to implement nationwide adoptive surveillance, like what is currently available for *Salmonella* Dublin, and a control strategy based on this data driven evidence. By far, probably due to no indication of serious zoonotic potential and apparently no visible economic impact of Q fever, the concerning authority in Denmark has not yet prioritised such a control program against this disease. However, recent outbreaks of Q fever in the Netherlands exhibited zoonotic consequences of this disease. Therefore, adoptive surveillance and a control program for Q fever in Denmark need to be implemented to mitigate such future emergency situations.

Epidemiological studies identified several risk factors associated with the prevalence and spread of *C. burnetii* infection in Danish cattle, thus also revealing that the infection load of *C. burnetii* is much higher in dairy cattle than beef cattle. Based on this acquired knowledge, an adoptive targeted surveillance can be implemented primarily focusing on dairy cattle. Several biosecurity measures were found to be associated with *C. burnetii* seropositivity. Therefore, implementation of strict biosecurity and legislative measures might be helpful for improving the situation. Strict veterinary legislative measures were found to be effective in controlling Q fever epidemics in the Netherlands (Roest et al., 2011). It is important to mention that the situation in Denmark is completely different from the Netherlands, as the recent epidemic in the Netherlands originated from small ruminants (Roest et al. 2011), whereas no such epidemic has been reported in Denmark.

## Conclusions

This thesis used data from two observational studies and has demonstrated the usefulness of such studies to draw a valid inference on the epidemiology of *C. burnetii* seropositivity in Danish dairy cattle. This thesis has presented epidemiological information on frequencies in different populations and the associated herd level and animal level risk factors of *C. burnetii* seropositivity in Danish cattle; these had not previously been studied thoroughly, and therefore the new developments have helped form a basis for further epidemiological investigations. The main conclusions which can be drawn from this thesis are:

- The estimated prevalence of *C. burnetii* antibody positivity in Danish dairy herds has increased since the latest survey in 2008, and is one of the highest among the European countries. High within herd prevalence of antibody positivity was also observed in dairy herds, and ranged from 1% to 27%. The overall true seroprevalence in cattle raised for meat production and sent to slaughter was 5.6%, with noticeable differences between dairy and beef breed cattle. The major proportion of the positive animals was attributed to the cattle of dairy breeds.
- Several herd management and biosecurity factors such as contact with farm personnel and visitors, a health agreement with the veterinarian, lack of hygienic precautions taken by the veterinarian and sharing of machines were associated with an increased risk of antibody positivity in Danish dairy herds. This stressed the importance of efficient farm management and strict biosecurity in preventing *C. burnetii* infections.
- The *C. burnetii* antibody status in Danish dairy cows was significantly associated with the individual animal factors like breed, parity, days in cow barn, milk yield, milk protein contents and the herd level factors like herd size, stable type, biosecurity related factors and season. Factors unravelled in Danish cows demonstrated similarities with factors identified in other countries, but some differences were also observed. These risk factors should therefore, be taken into account to prevent or control the introduction and spread of *C. burnetii*.

- Milk and blood ELISAs, the diagnostic tests used in the different studies included in this thesis, have similar diagnostic capabilities. Therefore, the choice of test should be based on the context of the study. For serological survey or surveillance involving dairy cows, milk ELISA should be the preferred method; and blood ELISA should be useful for serological studies in non-lactating cattle.

Using the findings of this thesis, pertinent bodies responsible for disease control and prevention could implement adoptive targeted surveillance and control strategies for *C. burnetii* infections in the Danish cattle population. The concepts and methodologies used in this thesis are generic and widely used in epidemiological studies of other infectious diseases as well.

## Perspectives

This thesis tries to form a basis to understand the epidemiology of *C. burnetii* infections in Danish cattle. However, in the light of new findings and targets to optimise the knowledge about the complete epidemiological profile of *C. burnetii* infection, further research needs to be done.

Small ruminants (e.g., sheep and goats) are important sources of *C. burnetii* infection abroad and have been involved in recent Q fever outbreaks. Further research is needed to investigate the burden of *C. burnetii* infection in small ruminants in Denmark.

Additional research is necessary to investigate the economic impact of *C. burnetii* infection in the Danish cattle population. *C. burnetii* infection is often said to be a cause of reproductive disorders. Information about reproductive problems available in the Danish Cattle Database is not systematically recorded, and therefore, the association between the infection status and reproductive problems cannot be evaluated based on this data. More data and systematic studies are required to investigate this association.

More large-scale studies on the effect of management and biosecurity factors on prevention and control of *C. burnetii* infection are necessary to validate the results found in this thesis. In addition, further studies of environmental contamination of *C. burnetii* and consequences of such contamination in epidemiology in Denmark are also needed.

Research is needed to explore the spatio-temporal distribution of *C. burnetii* infected/seropositive herds in Denmark. Such additional studies require a nationwide surveillance and could help to understand the distribution in terms of time and space.

The studies in this thesis identified *C. burnetii* antibodies in samples collected from individual animals or herds (BTM), thus providing an indirect measure of infection. Further studies based on identification of bacteria are needed to identify the actual burden of infection. Besides this, there is a need for additional research on simultaneous identification of antibodies and bacteria from repeatedly sampled herds and animals for a longer period. Such research will help to understand the infection dynamics and will also



be able to establish the relationship of the antibody status and the shedding pattern of the bacteria.

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**CHAPTER 8**

**ANNEX (QUESTIONNAIRE)**



## Questionnaire (English version)

# Q fever survey 2008

## Project I: Prevalence Survey

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**Questionnaire used in telephone interviews of dairy herd owner/manager for collecting herd level information.**

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**Employee:**

**Responsible:**

Jens Frederik Agger, Associate Professor, Department of Large Animal Sciences, Section for Population Biology (Epidemiology), LIFE, University of Copenhagen. Grønnegaardsvej 8, DK-1870 Frederiksberg C.

**Student Employee:**

Lærke Boye Thomsen, student of Veterinary Medicine.

*Remember that courtesy is a matter of course. We would indeed like to come back. ☺*

---

**Name of interviewer:**

Name: \_\_\_\_\_

---

**General information**

Date of interview : \_\_\_\_ / \_\_\_\_ / 2008

Project number (1-150) : \_\_\_\_\_

CHR number : \_\_\_\_\_

Telephone number : \_\_\_\_\_

Farmer's name / address : \_\_\_\_\_

---

**Introduction:**

Good afternoon / evening, my name is xxx.

I'm calling from the Faculty of Life Sciences, University of Copenhagen, where we conduct a study of Q fever in Danish dairy cattle herds. A few days ago the Dairy Board sent you a letter with an invitation to participate in this study.

1. Have you received this letter? (If NO, we need to explain why we called. Then they probably say YES). : YES ☐ NO ☐



2. Are you willing to participate in this study? : YES ☐ NO ☐

---

**If NO**

3. Are there any special reasons why you do not want to participate?

---

---

---

Sorry for the inconvenience and have a good day / evening.

---

**If YES**

**Thank you. You will be attended soon.**

We will shortly send you a plastic tube to take a milk sample from your bulk milk tank. The sample is to be send to the National Veterinary Institute, Technical University of Denmark.

In the letter, there will be instructions on how the sample must be taken and send to the laboratory. We will enclose a stamped addressed envelope.

I would like to ask you some questions about your farm. It will take 20-30 minutes.

---

## Questions about the herd

**About number of employees:**

4. How many people help in milking, feeding and caring for : Number:   
your animals (cows, heifers, old calves and young calves)?

**About stables and health of animals**

5. What type of housing do you use for the cows?

Tie stall house ☐ Cubicle house ☐ Deep bed house ☐

Further elaboration here:

---

6. Where do the cows calve in the herd?

Calving pen ☐ Tie stall ☐ Cubicle or deep bed ☐

7. Do you have a disease pen for sick cows?

YES ☐

YES, but it also serves as a calving pen ☐

NO ☐

8. Have there been changes in the pattern of disease in cows during the last 6 months compared with earlier times?

**Syndrom**

**YES**

**NO**

Abortions

☐☐

Stillborn calves

☐☐

Weak-born calves

☐☐

Retained placenta

☐☐

Endometritis

☐☐

Reproductive problems

☐☐

Other

☐☐

9. Have there been changes in the pattern of disease in old calves/heifers the last 6 months compared with earlier times?

YES ☐

NO ☐

If YES, please specify: \_\_\_\_\_

10. Have there been changes in the pattern of disease in young calves the last 6 months compared with earlier times?

YES ☐

NO ☐

If YES, please specify: \_\_\_\_\_

**Biosecurity procedures, including contact with other herds**

11. Have you purchased cattle to the herd during the last 12 months?

Number of purchased cows

Number of purchased heifers and old calves

Number of purchased young calves

12. How many suppliers have delivered the purchased cattle?

13. How many suppliers have a herd health advisory contract with the veterinarian?

Number

Don't know ☐

14. Do you buy cattle from the assembly sites (e.g. livestock auctions)?

YES ☐

NO ☐

15. Do you bring your animals to shows or other exhibitions?

YES ☐

NO ☐

16. Do you buy fodder from other herds?

YES ☐

NO ☐

17. Do you buy straw or bedding material from other herds?

YES ☐

NO ☐

18. How often you visit other herds or stables?

NEVER ☐

RARELY ☐

OFTEN ☐

19. How often do other farmers visit your herd or stables?

NEVER ☐

RARELY ☐

OFTEN ☐

20. Do you share machines with other herds?

YES, cattle trucks ☐

YES, field machines ☐

YES, Other ☐ Specify: \_\_\_\_\_

NO ☐

21. Who inseminates the cows in your herd?

Artificial insemination (AI) technician ☐

Veterinarian ☐

By own bull ☐

Other ☐

**If other**, please specify: \_\_\_\_\_

22. Who is (are) the herd veterinarian(s)?

Name : \_\_\_\_\_

23. Do you have a herd health agreement contract with your veterinarian?

YES ☐

NO ☐

24. What other people get in contact with your cattle?

Livestock consultant ☐

Truck driver ☐

Other ☐ Specify: \_\_\_\_\_

25. Do you bring cattle on pasture during summer?

YES ☐

NO ☐ (if No, go to question 27)

**26. If YES to question 24:**

Do your cattle come into physical contact with animals in neighboring fields (across a common fence)?

YES ☐

NO ☐

Describe: \_\_\_\_\_

27. Do you bring cattle on common pasture with cattle from other herds?

YES ☐

NO ☐

Describe: \_\_\_\_\_

28. Have you prohibited other people from entering your stable without your permission?

YES ☐

NO ☐

29. Do you require hygiene and biosecurity precautions before people enter your stable?

Yes ☐

NO ☐

**If Yes**, please describe: \_\_\_\_\_

30. Do you quarantine purchased supply cattle?

Yes ☐

NO ☐

Don't buy new animals ☐

Explain: \_\_\_\_\_

31. Which hygienic measures does the herd veterinarian take before entering the stable?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

32. What hygienic measures does the herd veterinarian take at the end of the visit?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

33. What hygienic measures does the AI technician take before entering the stable?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

34. What hygienic measures does the AI technician take at the end of the visit?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

35. What hygienic measures does the livestock consultant take before entering the stable?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

Does not enter the stable ☐

36. What hygienic measures does the livestock consultant take at the end of the visit?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

Does not enter the stable ☐

37. What hygienic measures does the truck driver take before entering the stable?

Nothing ☐

Change clothes ☐

Wash/change boots ☐

Does not enter the stable ☐

38. What hygienic measure does the animal consultant take at the end of the visit?

Nothing ☐ Change clothes ☐

Wash/change boots ☐ Does not enter the stable ☐

39. What hygienic measure do OTHER people take before entering the stable?

Specify who is (are) the OTHER(S): \_\_\_\_\_

Nothing ☐ Change clothes ☐

Wash/change boots ☐ Do not enter the stable ☐

40. What hygienic measure do OTHER people take when finishing the visit?

Nothing ☐ Changes cloths ☐

Washes/changes boots ☐

41. Do you disinfectant foot ware before entering the stable?

Yes ☐ No ☐

42. Do you in other ways take action to protect the herd against infection?

YES ☐ NO ☐

Explain: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

## Questionnaire (Danish version)

# Q-feber-undersøgelsen 2008

## Delprojekt II: Prævalens-undersøgelse

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**Spørgeskema til brug ved telefoninterview om deltagelse og om drift af besætninger.**

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### Medarbejdere:

#### Ansvarlig:

Lektor Jens Frederik Agger, Institut for Produktionsdyr og Heste, Sektion for Populationsbiologi (Epidemiologi), LIFE, Københavns Universitet. Grønnegårdsvej 8, 1870 Frederiksberg C. Telefon: 3533 3013. Mobil: 2021 1208.

#### Studertermedarbejder:

Stud.med.vet. Lærke Boye Thomsen. Telefon: 3284 9420. Mobil: 2031 6577

*Husk at høflighed er en selvfølge. Vi skulle jo gerne kunne komme igen. ☺*

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### Interviewer:

Navn: \_\_\_\_\_

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### Den udspurgte:

Dato for Interview : \_\_\_\_ / \_\_\_\_ / 2008  
Projektnummer (1-150) : \_\_\_\_\_  
CHR-nummer : \_\_\_\_\_  
Telefonnummer : \_\_\_\_\_  
Landmandens navn og adresse : \_\_\_\_\_  
\_\_\_\_\_

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### Introduktion:

Goddag / aften, mit navn er xxx. Jeg ringer fra Det Biovidenskabelige Fakultet ved Københavns Universitet (tidligere KVL eller Landbohøjskolen), hvor vi har startet en undersøgelse af Q-feber i danske malkekvægbesætninger. For få dage siden sendte Mejeriforeningen dig et brev med opfordring om at deltage i denne undersøgelse.

1. Har du modtaget dette brev? (Hvis Nej, må vi : JA ☐ NEJ ☐  
forklare. Så siger de sikkert JA).

2. Er du villig til at deltage i denne undersøgelse? : JA ☐ NEJ ☐

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**Hvis NEJ:**

3. Er der nogen særlige grunde til, at du ikke ønsker at deltage?

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Undskyld ulejligheden – og fortsat god dag/aften.

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**Hvis JA:**

**Tak for at du vil deltage.**

Vi vil inden længe sende dig et plastikglas til at udtage en mælkeprøve fra mælketanken. Prøven skal du sende til Veterinærinstituttet, Danmarks Tekniske Universitet.

I brevet vil der være instruktion om, hvorledes prøven skal udtages og sendes. Der vedlægges frankeret svarkuvert.

Jeg vil gerne have lov at stille nogle spørgsmål om din besætning. Det tager kun ca. 20-30 minutter!

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## Spørgsmål om besætningen

**Om antal medarbejdere:**

4. Hvor mange forskellige mennesker hjælper med til : Antal:   
malkning, fodring og pasning i øvrigt af dine kreaturer (køer, kvier o.a. ungdyr, og kalve)?

**Om stalden og sundhed hos dyrene**

5. Hvilken staldtype holder du køerne i?

Bindestald ☐ Sengestald ☐ Dybstrøelsesstald ☐

Kan evt. uddybes her

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6. Hvor kælder køerne i besætningen?

Kælboks ☐ Båsen ☐ Løsdrift ☐

7. Bruger du en sygeboks til syge køer?

Ja ☐

JA, men fungerer også som kælvningsboks ☐

NEJ ☐

8. Er der sket ændringer i sygdomsmønstret hos køerne de seneste 6 måneder sammenlignet med tidligere tider?

**Sygdom**

**JA**

**NEJ**

Aborter

☐☐

Dødfødte kalve

☐☐

Svagt fødte kalve

☐☐

Tilbageholdt efterbyrd

☐☐

Børbetændelse

☐☐

Reproduktionsproblemer

☐☐

Andet?

☐☐

9. Er der sket ændringer i sygdomsmønstret hos ungdyrene / kvierne de seneste 6 måneder?

JA ☐

NEJ ☐

Hvis JA, forklar: \_\_\_\_\_

10. Er der sket ændringer i sygdomsmønstret hos kalvene de seneste 6 måneder?

JA ☐

NEJ ☐

Hvis JA, forklar: \_\_\_\_\_

**Om smittebeskyttelse, herunder kontakt med andre besætninger**

11. Har du indkøbt kreaturer til besætningen de seneste 12 mdr?

Antal indkøbte køer

Antal indkøbte kvier og ungdyr

Antal indkøbte kalve

12. Hvor mange leverandører har leveret de købte kreaturer?

13. Hvor mange af leverandørerne har en sundhedsrådgivningsaftale med dyrlægen?

Antal

Ved ikke ☐

14. Køber du kreaturer fra samlesteder (f.eks. husdyrauktioner)?

JA ☐

NEJ ☐

15. Deltager besætningens dyr i dyrskue eller andre udstillinger?

JA ☐

NEJ ☐

16. Køber du foder fra andre besætninger?

JA ☐

NEJ ☐



17. Køber du halm/strøelse fra andre besætninger?

JA ☐

NEJ ☐

18. Hvor ofte kommer du på besøg i andre besætninger/stalde?

Aldrig ☐

Sjældent ☐

Ofte ☐

19. Hvor ofte kommer andre landmænd på besøg i din besætning/stald?

Aldrig ☐

Sjældent ☐

Ofte ☐

20. Deler du maskiner med andre besætninger?

JA, kreaturvogn ☐

JA, markmaskiner ☐

JA, andet ☐ Beskriv: \_\_\_\_\_

NEJ ☐

21. Hvem inseminerer besætningens kreaturer?

Kvægavlsforening ☐

Dyrlæge ☐

Egen tyr ☐

Andre ☐

**Hvis ANDRE,** Beskriv : \_\_\_\_\_

22. Hvem er besætningens dyrlæge?

Navn : \_\_\_\_\_  
\_\_\_\_\_

23. Har du indgået Sundhedsrådgivningsaftale (SRA) med din dyrlæge?

JA ☐

NEJ ☐

24. Hvilke andre personer kommer i kontakt med dit kvæg?

Kvægbrugskonsulent ☐

Vognmand ☐

Andre ☐

Beskriv: \_\_\_\_\_

25. Sender du kvæg på græs om sommeren?

JA ☐

NEJ ☐ (Hvis NEJ, Gå til spørgsmål 27)

**26. Hvis JA til spørgsmål 25:**

Kan dine kreaturer på græs komme i fysisk kontakt med kreaturer på tilstødende marker (fælles hegn)?

JA ☐

NEJ ☐

Beskriv: \_\_\_\_\_  
\_\_\_\_\_

27. Sender du kvæg på fælles græsgang?

Ja ☐

NEJ ☐

Beskriv: \_\_\_\_\_  
\_\_\_\_\_

28. Har du givet forbud mod at andre personer må gå ind i stalden uden din tilladelse?

JA ☐

NEJ ☐

29. Stiller du krav om hygiejne og smittebeskyttelse, når andre personer går ind i din stald?

JA ☐

NEJ ☐

Hvis JA, Forklar: \_\_\_\_\_

30. Bruger du karantænestald ved indkøb af nye kreaturer?

JA ☐

NEJ ☐

Don't buy new animals ☐

Forklar: \_\_\_\_\_

31. Hvilke hygiejneforholdsregler foretager dyrlægen før han /hun går ind i stalden?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler boots ☐

32. Hvilke hygiejneforholdsregler foretager dyrlægen når han/hun er færdig med besøget?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler boots ☐

33. Hvilke hygiejneforholdsregler foretager inseminøren før han/hun går ind i stalden??

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler ☐

34. Hvilke hygiejneforholdsregler foretager inseminøren når han/hun er færdig med besøget?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler ☐

35. Hvilke hygiejneforholdsregler foretager kvægkonsulenten før han/hun går ind i stalden?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler ☐

Går ikke ind i stalden ☐

36. Hvilke hygiejneforholdsregler foretager kvægkonsulenten når han/hun er færdig med besøget?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler ☐

Går ikke ind i stalden ☐

37. Hvilke hygiejneforholdsregler foretager vognmanden før han/hun går ind i stalden?

Intet ☐

Skifter kittel/tøj ☐

Vasker/skifter støvler ☐

Går ikke ind i stalden ☐

38. Hvilke hygiejneforholdsregler foretager vognmanden når han/hun er færdig med besøget?

Intet ☐ Skifter kittel/tøj ☐  
Vasker/skifter støvler ☐ Går ikke ind i stalden ☐

39. Hvilke hygiejneforholdsregler foretager ANDRE før han/hun går ind i stalden?

Hvem er ANDRE: \_\_\_\_\_

Intet ☐ Skifter kittel/tøj ☐  
Vasker/skifter støvler ☐ Går ikke ind i stalden ☐

40. Hvilke hygiejneforholdsregler foretager ANDRE når han/hun er færdig med besøget?

Intet ☐ Skifter kittel/tøj ☐  
Vasker/skifter støvler ☐

41. Bruges der desinficerende fodbad før indgang i stalden?

JA ☐ NEJ ☐

42. Bruger du andre tiltag for at beskytte besætningen mod smitte?

JA ☐ NEJ ☐

Forklar: \_\_\_\_\_  
\_\_\_\_\_